

Antidotal effects of thiol containing compounds
with a special study of
a method for the detection of BAL

by

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I. INTRODUCTION

As early as 1909 Ehrlich on the basis of previous studies, in particular those of Heffter and collaborators in 1904 and Heffter in 1908, stated his opinion that the toxic action of arsenic on living organisms is due to its attack on sulfhydryl (-SH) compounds or "arsenoceptors", which are essential to biologic oxidation and reduction processes. Many other investigators have advanced the hypothesis that metallic compounds and certain other poisons combine with the thiol groups of tissue enzymes or with those of members of oxidation and reduction system, such as glutathione and cystine, and thus inactivate those constituents essential to life and function. Long before the outbreak of the last war the concept was quite generally held that "detoxification" of arsenic, as well as of certain other metallic and even non-metallic poisons, may be accomplished by chemicals containing thiol groups. One of the early fundamental studies of this effect was that of Voegtlin et al (1923) who demonstrated that the toxic action of arsenic (in the form $R.As=O$) was due to its reaction/

reaction with the -SH group of glutathione and other possible -SH compounds which may occur in protoplasm. They showed that reduced glutathione, thioglycollic acid and other simple monothiols counteract the toxic action of arsenoxide (3-amino-4 hydroxyphenyl arsenious oxide) on trypanosomes both in vitro and in the circulating blood of infected rats. The corresponding oxidised disulfides (R.S.-S.R.) of these monothiols were much less effective or practically without effect, and similar aminoacids containing no -SH group possessed no anti-arsenical action. In their paper it was further demonstrated that the toxic action of arsenoxide on rats was diminished by a previous injection of thioglycollate, as shown by a longer survival in the treated group than in the controls receiving arsenic alone. In 1925 the same workers reported that a prophyl/actic injection of reduced glutathione would protect rats against the lethal effect of arsenoxide. It was necessary to maintain a ratio of 10 mols. of glutathione to 1 mol. of arsenoxide in order to afford protection. Reduction in mortality and some prolongation of survival time were effected by treatment with reduced glutathione in similar molar ratio, twenty/

twenty minutes after administration of arsenoxide. Rosenthal and Voegtlin (1930) reconfirmed the beneficial action of previously administered crystalline glutathione, thereby eliminating the possibility that the protection had been afforded by an unknown contaminant in the earlier glutathione preparation.

The protective action of monothiols against the toxic action of arsenic on biological systems was confirmed by both in vitro and in vivo experiments. Voegtlin et al (1931) showed that glutathione, in a ratio of 10 mols. to one of arsenoxide, could prevent the reduction in O_2 consumption caused by the addition of the latter drug to rat liver, kidney and testis slices. These workers also showed, as had Rosenthal and Voegtlin (1930), in both trypanosomes and rats that when sodium arsenite was used instead of arsenoxides, 40 mols. of glutathione were required to protect against 1 mol. of arsenite. Walker (1928) showed that the addition of monothioethylene glycol to protozoa three minutes after the administration of 3 M.L.D. of diphenylchlorarsine brought about rapid revival of the organisms, although death followed in 1 to 2 hours. Schmitt and Skow (1935) demonstrated that monothiols delayed the extinction of the nerve action/

action potential produced by arsenite on medullated nerves of frogs, but that regardless of the ratio of SH to As, monothiols were unable to prevent the eventual disappearance of the action potential.

Strangways (1937) while investigating the trypanocidal action of various thioarsenites, found that, in vitro in dilute solution, the lethal activities of a thioarsenite (salvarsan oxide glutathione) and its parent oxide in equimolar concentrations are identical, whereas in strong solution the oxide is of greater toxicity. She also demonstrated that, whereas in strong solution an excess of glutathione inhibits the lethal action of both an arsenoxide and a thioarsenite in high dilution, even with a 10:1 molar excess of glutathione, protection is not afforded. It appears that in strong solutions the excess of glutathione favours the formation of thioarsenite and that in more dilute solutions dialysis occurs with the liberation of toxic arsenoxide.

Experiments carried out in the department of Biochemistry at Oxford by Sinclair (1940), indicated that none of the large series of monothiols was able to protect the brain pyruvate oxidase system against lewisite./

lewisite. The contrast between the favourable results of earlier workers with aromatic therapeutic arsenoxides, and the failure of thiols to protect against lewisite or arsenite, was thought to depend largely on differences in the degree of dissociation of the thio-arsenite formed. Stocken and Thompson (1941) brought forward the idea that the high toxicity of lewisite in particular and of the trivalent arsenicals in general might be due to their ability to combine with essential -SH groups in certain tissue proteins to form stable arsenical rings. They pointed out that on chemical grounds

compounds of an $\begin{array}{c} \text{R} \\ | \\ \text{R} \end{array} - \begin{array}{c} \text{S} \\ | \\ \text{S} \end{array} \rangle \text{AsR}'$ would be expected

to be more stable than compounds of an arsenical with two molecules of mono-thiol, i.e., of the

type $\begin{array}{c} \text{R} \\ | \\ \text{R} \end{array} - \begin{array}{c} \text{S} \\ | \\ \text{S} \end{array} \rangle \text{AsR}'$. For these reasons, therefore, it was felt that simple dithiol compounds might form relatively ring compounds with lewisite or other trivalent arsenicals and might therefore, be more effective than the monothiols in protecting against arsenic. These workers prepared a group of dithiols which/

which included 1-2 dimercaptopropanol (BAL), 1-3 dimercaptopropanol, 1-3 dimercaptopropane, and 1-2 dimercaptoethane; compounds which they anticipated, on the basis of physical and chemical properties, would be effective in protection of biological systems against arsenic. They later on showed that these compounds in low molar ratios were effective in preventing inhibition of pyruvate oxidase caused by lewisite. At a molar ratio of 4 dithiol to 1 As., some protection was afforded by all these compounds. BAL (1-3 dimercaptopropanol) at a molar ratio of 8.5 to 1 caused complete protection of the system, and this substance alone was not toxic to the enzyme in concentrations of 27×10^{-5} M.

Later on Thompson and Stocken (1941), Stocken et al (1942), and Peters et al (1943) demonstrated, by their original experiments, the great effectiveness of the dithiol BAL in saving animals systematically poisoned by liquid lewisite and showed the unequivocal benefits effected when BAL was externally applied to skin contaminated by lewisite. After these facts had been established, many other investigators - first in England and then in America - turned their efforts, not only to further researches but/

but also to the immediate practical problems of large scale production and standardisation of BAL and the development of its practical uses in the prevention and treatment of lewisite and other arsenical damage.

Inspite of so much investigation and research, no reliable method for estimating BAL in vivo has, as far as is known, yet been described. An obvious possible method consists of using the reversal of the poisoning of a suitable enzyme by some heavy metal, provided that a consistent relation can be found between the concentration of BAL and the degree of reversal. At the suggestion both of Professor R.A. McCance and Dr. L.A. Stocken, the possibility of using mercury and arsenic poisoned urease was explored. A method for estimating BAL in vitro and in vivo has been worked out on this line which has been described in the next chapter.

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II. Methods

(a) Principle

The method described for the estimation of thiols is based on their ability to prevent the inhibition of urease by salts of mercury. The activity of urease has been measured in Conway's micro-diffusion units by estimating the amount of ammonia produced in a standard time from an excess of urea.

(b). Technique of experiment.

It is described in two parts -

- (i) technique for in vitro experiment and
- (ii) technique for experiment in vivo.

(i) In vitro experiment:- The estimation of urea was done by the method of Conway and O'Malley (1942) with minor modifications. 2 ml. of Boric acid buffer was run into the central chamber of each Conway unit. In the outer chamber of each unit, 0.2 ml. of 120 mg.% urea was measured and a thin coat of vaseline was applied on the outer rim of the units. Dilutions of urease were prepared in 5 ml. volumetric flasks, into each of which was measured 1 ml. of concentrated urease solution and varying quantities of HgCl_2 and BAL. The flasks were then filled up to the mark with distilled water, shaken and allowed to stand for about 2 minutes. A volume of 0.5 ml. from the flasks was then measured with a micro-pipette into the outer chamber of the Conway units. The units/

units were then sealed and left at room temperature for 30 minutes. A volume of 1 ml. of a saturated solution of K_2CO_3 was added to the outer chamber. The unit was then again sealed and left at room temperature for 1 $\frac{3}{4}$ hours. The fluid in the central chamber was then titrated with N/200 Hcl. Such experiments were carried out in duplicate or triplicate.

(ii) In vivo experiment:- For in vivo experiment throughout the investigation, adult rabbits, male and female, were used. The average weights ranged from 1.1 to 2.1kg. All were kept on the standard laboratory diet throughout the experimental period and were given water ad lib. The drugs were always injected intramuscularly in the muscles on the back. The blood was withdrawn from the ear veins and heparinised. Urine was collected either in a metabolism cage or the animal was catheterised by No.3 rubber catheter.

(c) Expression of result.

If the amount of urea hydrolysed by a given amount of urease was 'a' ug., the amount hydrolysed in the presence of a heavy metal 'b' ug., and the amount in the presence of a heavy metal and a thiol 'c' /

'c' μ g; the heavy metal poisoning was expressed as $100 \frac{a - b}{a} \%$ of the normal urease activity and the antimetallic activity of a thiol was $100 \frac{c - b}{a - b} \%$ of the inactivated urease.

(d) Urease enzyme.

Until recently, studies on urease were made with more or less crude preparations derived from defatted soya or Jack bean meal. The preparation of Vanslyke and Cullen (1914), admirable as reagent catalyst in estimations of urea, contained impurities which masked certain properties of the enzyme itself.

Conway (1939) prepared a urease solution by extraction from Jack bean meal with glycerine after the manner described by Schmidt (1928). He found this solution to be very potent and kept well for months under refrigeration.

Kingley (1944) observed that urease solutions prepared by extraction of Jack bean meal with alcohol and water were not stable at room temperature and when refrigerated may not be kept safely for more than one month. The alcohol of these extracts may also decrease the stability of the colour developed/

developed after nesslerization. Water solutions of commercial urease powder and also of Jack bean meal or soya bean flour are also unstable. He devised a method for the preparation of a more stable concentrated urease solution from Jack bean meal or from a commercial urease powder.

Grant and Kinsay (1946) used purified crystalline urease for assessing the influence of pH, phosphate, alkylating agents etc. They prepared crystalline urease after the method described by Sumner (1926). Sumner's method however, requires a very long time and is extremely elaborate. Moreover, it has been reported (Oppenheimer, 1926; Jacoby, 1928 and 1933; Kitagawa, 1929; and Ruckelman, 1933) that urease, even in crude mixtures, is sensitive to the action of a number of salts, e.g., those of silver, mercury and copper.

In view of these facts an attempt was made to use the water extract (fresh preparation) of soya bean flour, 1 gm. to 5 ml. of distilled water; shaken well and centrifuged. The clear supernatant fluid was used as the urease enzyme. This preparation was tried several times but no constant results could be obtained. So extraction of urease with glycerol/

glycerol from soya bean flour was tried according to Conway (1939) with some modification as described below.

Preparation of urease.

22 gm. of finely powdered permutit was washed with 2% acetic acid, which was then decanted off. The permutit was subsequently washed twice with distilled water. This washed permutit was mixed with 90 gm. of finely powdered soya bean flour and 500 ml. of distilled water. These were shaken for 15 minutes and then 225 ml. glycerol added and mixed. The whole was allowed to filter overnight. The filtrate was collected and stored in a refrigerator in a stoppered bottle. In these conditions it retained its activity at least for 3 months.

Ammonia production.

The activity of this urease preparation was observed at different dilutions when allowed to act on different quantities of urea for different lengths of time. The results are shown in Tables 1 and 2.

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Table 1/

TABLE 1

Quantity of hydrolysis of urea with different concentrations of urease when urease was allowed to act for 15 minutes.

Ug. of urea added to unit.	Concentrations of urease used.		
	Undiluted	1:5	1:10
60	60 µg.	48 µg.	22.5 µg.
120	120 µg.	120 µg.	-
180	180 µg.	-	-
240	202.5 µg.	-	-

TABLE 2

Quantity of hydrolysis of urea with different concentrations of urease when allowed to act on 240 µg. of urea for different lengths of time.

Concentration of urease	5'	10'	15'	20'	30'	60'
1:5	-	-	60µg.	-	105µg.	-
1:10	15ug.	24µg.	42 µg.	43.5 µg.	73.5µg.	96µg.
1:20	-	-	-	-	27µg.	-
1:100	-	-	4.5µg.	-	4.5µg.	-
1:1000	-	-	nil.	-	nil.	-

These tables show that (1) with undiluted urease, the ammonia produced in 15 minutes was equivalent to the urea present up to 180 μ g. Above this level hydrolysis was incomplete. (2) With a 1:5 dilution of the urease extracts used and 240 μ g. urea, ammonia produced in 30 minutes was 105 μ g. (39.1%) urea and with more dilute urease, there was less hydrolysis down to 4.5 μ g. (1.9%) urea with 1:100 and nil with 1:1000. Up to 30 minutes the rate of hydrolysis was roughly linear and the limiting factor was the amount of urease present. So this procedure, of allowing 1:5 dilution of urease to act for 30 minutes on 240 μ g. of urea, was adopted throughout the investigation.

Two such preparations were tried during this investigation from the first sample of soya bean flour. Afterwards when it exhausted, several market preparations of soya bean flour were tried, but none gave a satisfactory yield. So B.D.H. urease tablets each of 50 mg. urea capacity were used. These tablets were also worked out to give similar results. Five of these tablets when dissolved in 100 ml. of distilled water were equivalent to the glycerol extract of soya bean flour. Three such/

such preparations of tablets were used in this investigation. These were also stored in the refrigerator. Lastly, soya Bean flour B.D.H. was used. The glycerol extract of this soya bean flour was found more potent when a similar preparation was made. 1:50 dilution of this glycerol extract was found to give a similar result. Four preparations of this soya bean flour (B.D.H.) were used in the investigation. These glycerol extracts were also stored in the refrigerator.

As the work progressed, it was found that the activity of urease fluctuates with the temperature and pH of the solutions. This observation was in accord with Vanslyke and Cullen (1914); Vanslyke and Zacharias (1914); and Conway (1939). So in the following experiments the pH of all the solutions used were maintained between pH 7 to 7.5 (as tested with universal indicator) by the addition of phosphate buffer of Sørensen (1912), and every time the temperature of the room was noted during each set of experiment. A statement showing the effect of temperature on different preparations of urease has been given in Table 3.

TABLE 3/

TABLE 3.

Quantity of hydrolysis with 240 µg. of urea with different preparations of urease under the influence of varying temperatures.

Source of urease	Prepara- tions used.	Dilu- tions	µg. of urea hydrolysed.						
			6 to 8°C.	8 to 10°C.	9 to 11°C.	10 to 12°C.	11 to 13°C.	12 to 14°C.	13 to 15°C.
1st sample soyabean	31.10.46	1:5	-	-	94.5	95.25	-	-	-
	18.2.47		75	-	95.25	96	-	-	100.5
B.D.H. tablets.	18.4.47		-	-	-	-	90.75	100.5	104.25
	5.5.47	1:5	-	-	-	-	-	-	104.25
	21.5.47		-	-	-	-	-	117	122.25
B.D.H.	16.6.47		-	-	-	-	-	117.75	123
Soya	18.7.47	1:50	-	-	-	-	-	117	124.5
bean	20.8.47		-	-	-	-	-	98	115.5
flour.	24.10.47		-	78	-	87.5	-	94.5	100.5

TABLE 3 Continued on following page - page 17.

TABLE 3. - (Continued)

Quantity of hydrolysis with 240 µg. of urea with different preparations of urease under the influence of varying temperatures.

Source of urease	Preparations used.	Dilutions	µg. of urea hydrolysed.					
			14 to 16°C.	15 to 17°C.	16 to 18°C.	17 to 19°C.	18 to 20°C.	19 to 21°C.
1st sample soyabean	31.10.46	1:5	-	-	-	-	105	-
	18.2.47		-	109.5	-	-	112.5	-
B.D.H. tablets	18.4.47		123.75	-	-	-	-	-
	5.5.47	1:5	114	135	-	-	-	-
	21.5.47		125.25	135.75	-	144	150	-
B.D.H.	16.6.47		135	139.5	143.25	149.3	-	-
Soya	18.7.47	1:50	127.5	-	138	144	-	-
bean	20.8.47		-	132	145.5	147	150	168
flour	24.10.47		108	112.5	-	-	-	-

Table 3 shows that the ammonia produced in 30 minutes with 240 μ g. of urea varied from 75 μ g. to 168 μ g. (32 to 70%) of the total urea activity according to the temperature and preparations of urease used irrespective of the source from which the urease enzyme were obtained.

(e) Poisoning of urease by metallic ions

The poisoning action of certain salts e.g., AgNO_3 , HgCl_2 upon enzymes is a well established fact. Euler and Svanberg (1920) reported the inhibiting effect of silver ion upon saccharase. Schmidt (1928) studied the inactivating power of various salts on urease, and suggested the possibility of its standardisation with mercuric chloride with the use of colorimetric method. He used urease prepared from Jack bean meal after the method of Koch (1926), which was essentially a modification of the old glycerol preparations of Jensen (1915), Robinson and Oppenheim (1919), and Wester (1920). Just prior to its use the concentrated glycerol urease was diluted 1:100 with distilled water. He observed that 0.002 to 0.004 mg. of mercuric chloride was essential to inactivate the diluted urease ~~inactivated~~. Other metals such as copper, zinc, cadmium, uranium/

uranium, gold etc., then followed order in that of decreasing activity. These results were in general accord with the findings of other investigators using different methods, viz. Vanslyke and Zacharias (1914), Falk and Sugiura (1914), Wester (1922), Jacoby (1916, 1920, 1922 and 1923), Loeb and Bodansky (1927) and Hosokawa (1924).

Hellerman and Newman (1932), and Hellerman, Perkins and Clark (1933) treated the potent preparations of crystalline urease in appropriate solutions with a series of organo-mercurials of the type $R.Hg.X$, including phenyl mercuric chloride or hydroxide; benzyl-mercuric chloride or chloromethylmercuric chloride ($ClCH_2HgCl$). In all these cases they observed that the ureolytic activity was rapidly inhibited by the presence of mercuric ions in the various solutions used.

Hellerman, Clinard and Deitz (1943) while studying the effect of protein sulphhydryl groups and the reversible inactivation of the enzyme urease, could inactivate the ureolytic activity of crystalline urease with p-chloromercuribenzoate. They have reported in their paper that a definite amount of mercury reagent must be added before the inactivation of/

of urease begins. The inactivation is complete only after a second Stoichiometrically comparable portion of p-chloromercuribenzoate has been used up.

In the present work two heavy metal salts were used for inactivating the urease preparations. These were HgCl_2 (mercuric chloride) and As_2O_3 (anhydrous arsenous oxide).

Mercuric chloride.

Aqueous solution of mercuric chloride of 0.125mM strength was prepared by dissolving 338 mg. of HgCl_2 in a litre of distilled water. From this stock solution different quantities of mercury were added to a 1:5 dilution of urease preparation of 31.X.'46 and were allowed to act on urease from 0 to 30 minutes, before the mixture was added to urea. The results obtained are given in Table 4 and Figure 1.

Similar previous assessment of the quantities of mercury required to produce 70 to 80% inhibition of urease activity was made in each case of different preparations of urease. The quantities of mercury varied with the different preparations of urease as shown in Table 5.

TABLE 4/

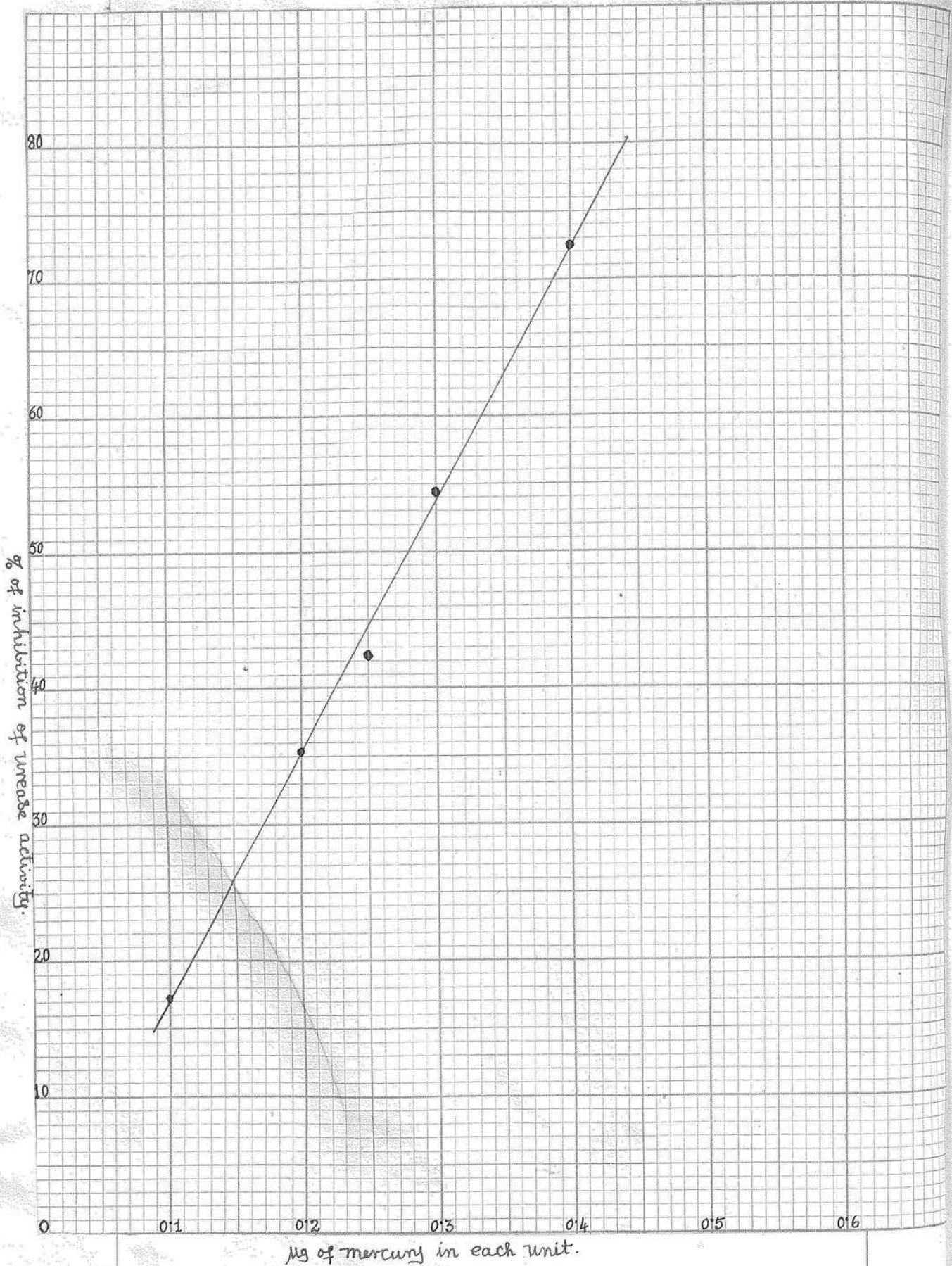


Figure I.

Showing the % of inhibition of urease (31.3.46) with mercury when allowed to act for 0 to 1 minute.

[To face page 21].

TABLE 4

% of inhibition of urease activity (31.X.47)
due to poisoning with different concentrations of
mercury when allowed to act for different lengths
of time before estimating the activity.

Amount of mercury added to each unit.	Time in minutes			
	0	10	20	30
0.1 µg.	17.1	-	-	-
0.2 µg.	35.1	-	-	-
0.25 µg.	42.8	-	-	-
0.3 µg.	54.3	-	-	-
0.4 µg.	72.3	72.1	72.2	72.1
0.5 µg.	92.3	92.1	92.1	-
1 µg.	complete	-	-	-
10 µg.	complete	-	-	-
100 µg.	complete	-	-	-

TABLE 5/

TABLE 5

% of inhibition of urease activity due to poisoning with different concentrations of mercury on the different preparations of urease when allowed to act for about one minute. (— These % of inhibition were used for reversal activity by thiols).

Preparations of urease used.	Amount of mercury added to each unit.									
	0.1μg.	0.2μg.	0.25μg.	0.3μg.	0.4μg.	0.5μg.	1μg.	10μg.	100μg.	
30.X.46	17.1	35.1	42.8	54.3	72.1	92.1	com-plete	com-plete	com-plete	
18.II.47	-	-	-	-	59.1	77.4	-	-	-	
18.IV.47	-	-	36.7	57	77.6	92.5	-	-	-	
5.V.47	-	-	-	-	75.9	-	-	-	-	
21.V.47	-	-	-	-	75.6	-	-	-	-	
16.VI.47	-	-	-	75.6	96.1	com-plete	-	-	-	
18.VII.47	-	66.8	80.9	95.1	com-plete	-	-	-	-	
20.VIII.47	-	38.6	-	80.3	95.1	com-plete	-	-	-	
24.X.47	-	-	37.3	74.8	89.3	-	-	-	-	

0.25 to 0.5 μg . of mercury in each unit was required to inhibit 70 to 81% of urease activity according to the different preparations of urease used. The % of inhibition by mercury in these conditions was not altered if they were allowed to react for up to 30 minutes before adding to urea (Table 4). So in all the experiments a time interval of 0 to 1 minute was allowed for Hg to act on urease before it was added to urea.

In each case, as is shown in Table 4 and Figure I, the inhibition of urease was proportional to the amount of mercury present in the solution.

Anhydrous arsenious oxide.

An aqueous solution of 0.125 mM of arsenious oxide was prepared as follows.

124 mg. of As_2O_3 (anhydrous arsenious oxide) was dissolved in 4 ml. of 2N sodium hydroxyde. 5.7 ml. of 5% Hcl (1.4 N Hcl) was added to it to neutralise the excess of NaOH. 20 ml. of phosphate buffer pH 7.2 (prepared from A.R. reagents) was added to it and the final volume was made up to a litre. This solution of arsenite contained 93.75 As/ml. (0.125 mM).

An attempt was made to poison the ureolytic activity/

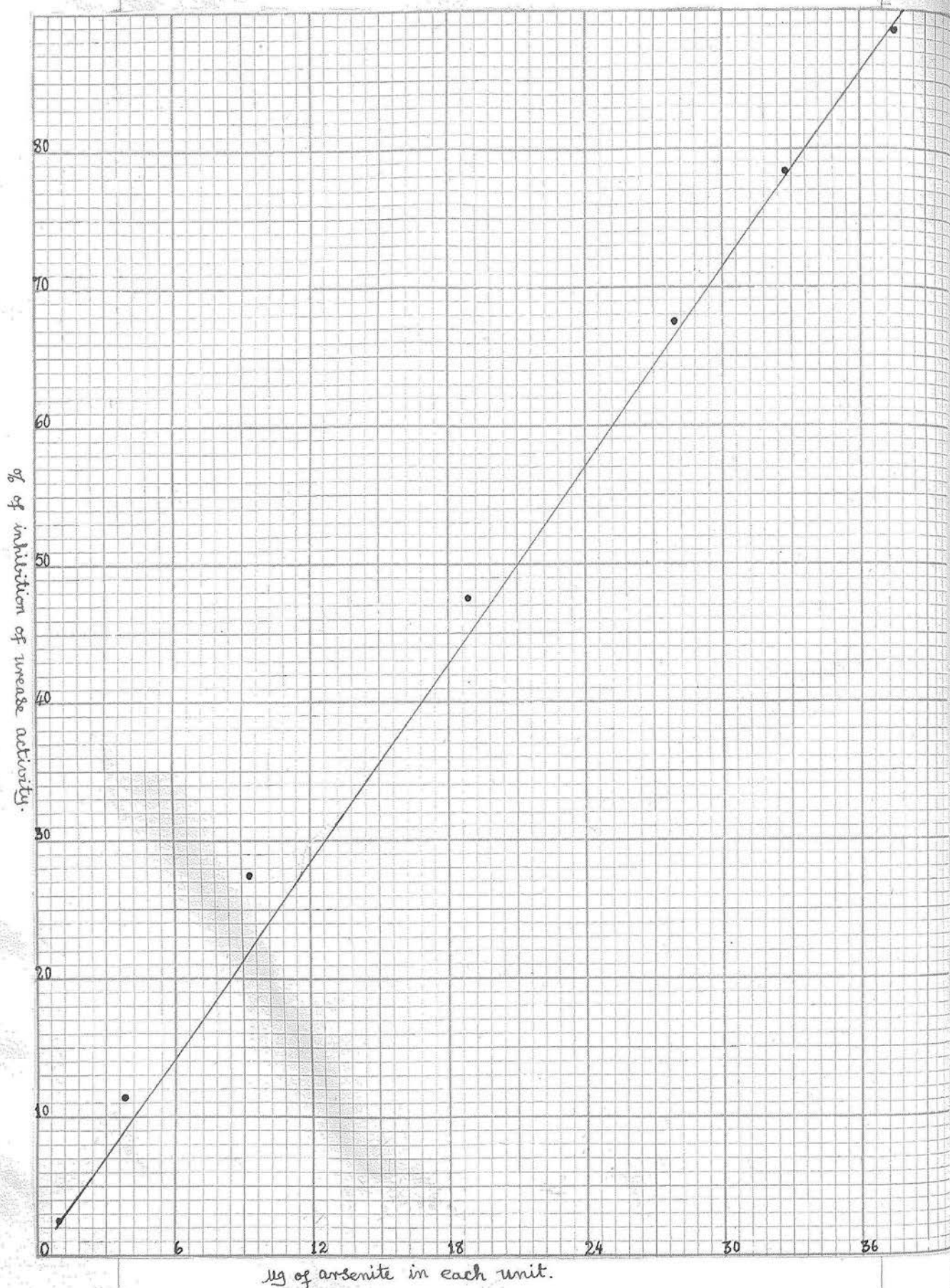


Figure II.

Showing the % of inhibition by arsenite
when allowed to act on urease (21.V. '47)
for 0 to 1 minute.

activity of urease up to 70 to 80% of its total activity by adding different quantities of the 0.125 mM arsenite solution as in the case of mercuric chloride solution. In this case, only 0 to 1 minute time was allowed for reaction between arsenite and 1:5 fresh urease dilutions. The results obtained are shown in Table 6 and Figure II.

TABLE 6

% of inhibition of urease activity due to poisoning with different concentrations of arsenic on urease preparation of 21.V.47, when allowed to act for 0 to 1 minute before the estimation was made.

Amount of arsenic in μ g. present in each unit.							
0.47	0.94	3.75	9.37	18.75	28.12	32.81	37.5
Nil	2.8	11.3	27.5	47.5	67.6	78.2	88.6

From Table 6 and Figure II, it is clear that the % of inhibition of urease is proportional to the amount of arsenic present in the solution, but the amount of arsenic required to inhibit about 70 to 80% of the urease activity is much more in comparison to/

to mercury under similar conditions. Tables 5 and 6 show that the same preparation of urease (21.V.47) requires 0.4 μ g. of mercury to inhibit about 75.6% the urease activity, which under the same condition needs 32.81 μ g. of arsenic for nearly the same amount of inhibition, i.e., mercury is about 82 times more potent in comparison to arsenic for inactivating the urease activity.

(f) Disinhibition of poisoned urease.

As early as in 1920 it was shown by Euler and Svanberg that the inhibiting effect of silver ion on succharase was fully reversed by H_2S . Soon after this Sumner (1932) demonstrated the reversal of poisoning of crystalline urease by H_2S .

Hellerman and Perkins (1934) showed that the urease activity was extinguished by the treatment of the enzyme with cuprous oxide or certain organic mercurials. This effect was likewise demonstrated to be reversible due to the formation of mercaptide.

Hellerman (1937) introduced p-chloromercuribenzoic acid (Hg-cl-benzoate) as an inhibitor of -SH enzymes; the inhibition produced by it was easily reversed on the addition of thiols (cysteine and glutathione)./

glutathione). In spite, however, of the knowledge, some heavy metals formed mercaptides, very little has been done to see if they combine with the -SH groups of the proteins.

Since the discovery of BAL as an arsenical detoxicant (Stocken and Thompson, 1940, 1941; Peters, Stocken and Thompson, 1945), research has been in progress in relation to the inactivation of certain enzymes by various heavy metals and then reactivation of the same by different thiols.

The reactivation by the action of cysteine upon urease inactivated with p-chloromercuribenzoate was secured without exception by Hellerman, Chinard and Deitz in 1943. They obtained complete restoration of the activity of urease. Frequently the activity was slightly greater than the initial and even impure urease of sufficient potency could be titrated back.

Stocken, Thompson and Whittaker (1947), using the pyruvate oxidase system of brain, have shown that BAL can protect effectively against the toxic action of mapharside, neoarsphenamine and arsphenamine, and can also bring about a significant degree of/

of reactivation of the already poisoned enzyme system.

Barron, Miller, Bartlett, Meyer and Singer (1947), using the pyruvate oxidase system as enzyme and lewisite as poison, found that in every case enzyme inhibition was reversed by the addition of BAL which was found to be a better reversal agent than glutathione.

Barron and Kalnitsky (1947) while studying the inhibition of succinoxidase by heavy metals and its reactivation with dithiols reported that a large number of heavy metals has been found to inhibit the enzyme activity of succinoxidase. The inhibition is due to the combination of the metal with the -SH groups of the protein moiety. Bi, Cd, HgCl_2 had the greatest inhibitory power, producing 90% inhibition at $c.10^{-5}$ M concentration. V, Zn, and Pb produced effective inhibitions at about 10^{-4} M; Zn and Sb required higher concentrations, half inhibition being attained at about 1.5×10^{-3} M. The inhibition produced by these heavy metals was reversed on addition of dithiols. The most effective compounds for the reversal of Bi, Cd, and Hg inhibition were 1:3 propane dithiol; 1:3-dimercaptopropanol and 2:3-dimercaptopropionic acid. Reversal by BAL (2:3/

(2:3 dimercaptopropanol) occurred only at higher concentration. Glutathione at concentrations used with dithiols was generally ineffective; at higher concentration of inhibitor it produced reactivation. Reversal of inactivation became increasingly difficult when the concentration of inhibitor was raised so as to produce complete inhibition.

In these experiments urease either in the form of glycerol extract of soya bean or urease tablets, each of 50 mg. urea capacity, were used as enzyme and mercury and arsenic were the heavy metals used for inactivating the urease activity (vide supra). In each case, the amount of heavy metal (Hg or As) was found out which produced about 70 to 80% inhibition of the total urease activity (vide Tables 5 and 6). The following thiols were studied as regards their power to reverse poisoning of urease.

(i) Dithiols:

- (a) 2:3-dimercaptopropanol (BAL)
- (b) Mannitol-6-BAL ether.

(ii) Monothiols:

- (a) Thioglycollic acid.
- (b) Cysteine hydrochloride.

(iii) Triacetyl BAL.

(iv) l-cystine.

(ia)/

(i.a) 2:3-dimercaptopropanol (BAL):

Every day fresh aqueous solution of BAL (0.08m/100ml. x 1/100ml) was prepared before the experiment. Different amount of this solution was added to 1:5 dilution of urease which had been inactivated by the known amount of Hg. This mixture was allowed to stand at room temperature for about 2 minutes before it was added to urea. This method was adopted separately in each case with different preparations of urease used and the results obtained are represented in Table 7 and Figure III.

TABLE 7/

% of reversal of mercury poisoned urease.

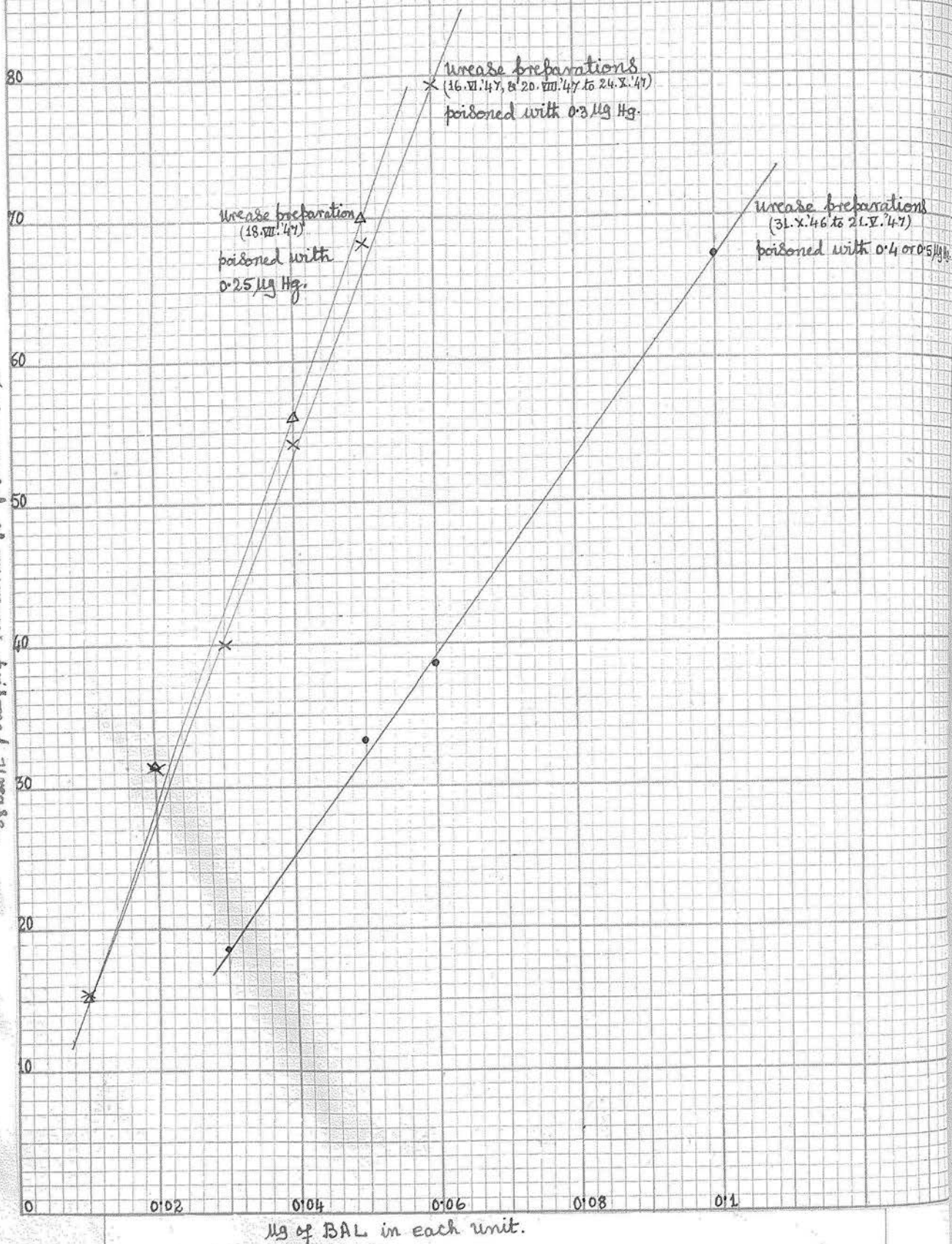


Figure III.
Showing the % of reversal with BAL
on mercury poisoned urease.

Reversal effect of 2:3-dimercaptopropanol (BAL) on the poisoned urease with known amount of Hg added to each unit and allowed to act for about one minute.

Preparation of urease used.	Ug. of Hg added to each unit	% of inhibition and Range	Ug. of BAL added to each unit.						
			0.01	0.02	0.03	0.04	0.05	0.06	0.1
31.X.46	0.4	72.1 (70 to 74.2)	-	-	18.8%	-	-	38.7%	-
18.II.47	0.5	77.4 (75.4 to 80)	-	-	-	-	33%	-	67%
18.IV.47	0.4	77.6 (75.3 to 80.4)	-	-	-	-	33.3%	-	67.2%
5.V.47	0.4	75.9 (72.7 to 78.4)	-	-	-	-	33.2%	-	67.3%
21.V.47	0.4	75.6 (73.4 to 77.8)	-	-	-	-	33.4%	-	67.1%
16.VI.47	0.3	75.6 (70 to 80)	15.2%	31.9%	-	54%	-	79.3%	-
18.VII.47	0.25	80.9 (80 to 83.3)	15%	31.6%	-	56%	70%	-	-
20.VIII.47	0.3	80.3 (79 to 82.9)	15.4%	31%	-	54.4%	-	79.6%	-
24.X.47	0.3	74.8 (70 to 79.3)	15.4%	-	40%	54%	68.2%	80%	-

From the foregoing table and figure it is evident that BAL when added within one minute after mercury reduced the inhibition. This reduction or reversal phenomenon was proportional to the amount of BAL present in the range of 0.01 μ g. to 0.1 μ g. according to the amount of mercury used for producing 70 to 81% of the inhibition. This reversal action was not influenced by the different preparations of urease used. The % of reversal with BAL at 70 to 81% inhibited urease activity had molar relation with Hg i.e., Hg: BAL::approximately 1:0.5 mM. This relationship of mercury and BAL is in accord with the result of Farah and Maresh (1948). They, employing the diuretic test in experimental animals, found that about half-a-mol of BAL is required to prevent or abolish the diuretic effect of one mol of mersalyl.

In the beginning, different concentrations of BAL (which were used for reversal action) were allowed to act on 1:5 dilutions of urease to see whether they had any inhibitory or activating action on urease itself and it was found that BAL did not affect the urease activity in quantities used for reversal of mercury inhibition in vitro experiments. The/

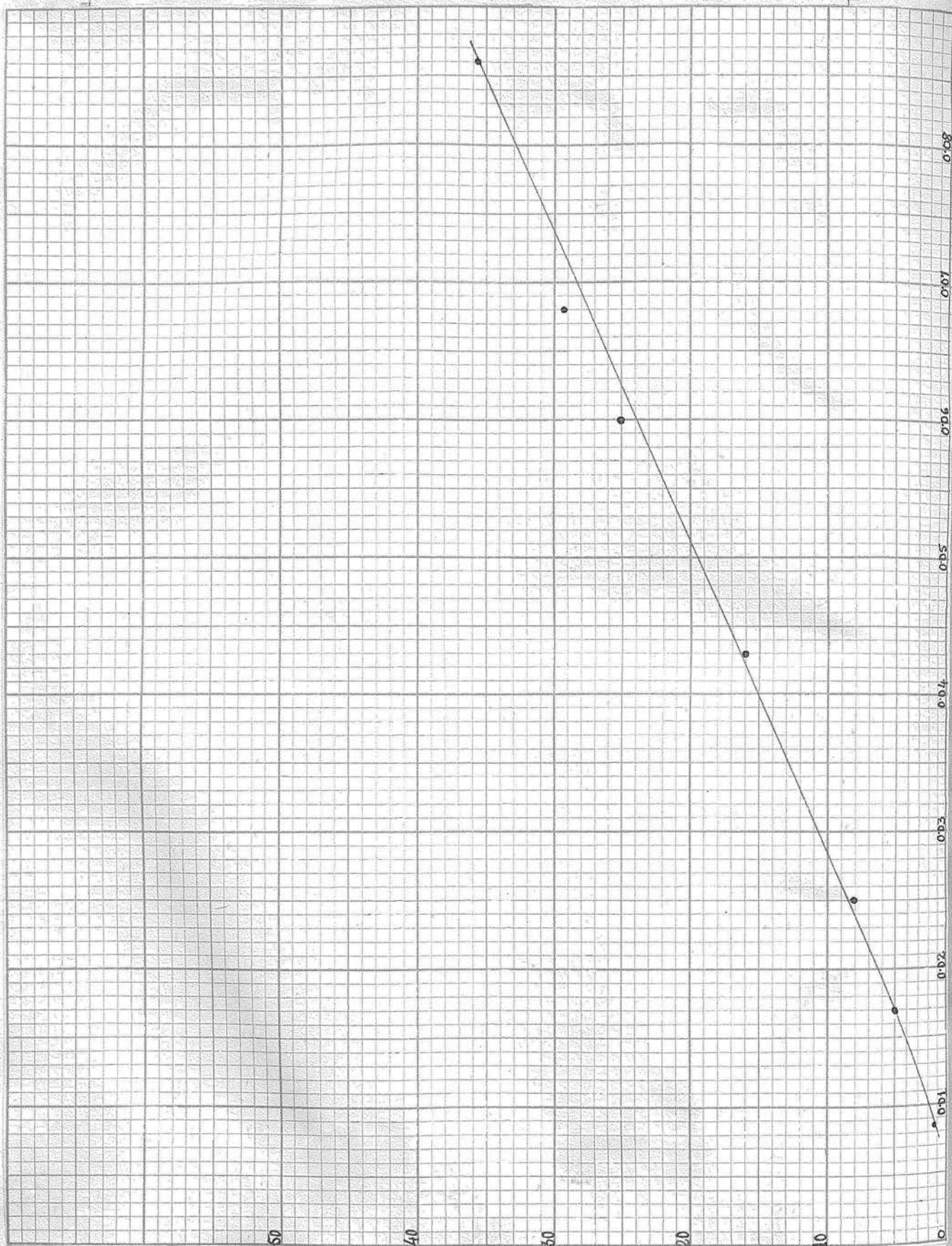
The reversal effect of BAL on mercury poisoned urease (i.e., where mercury was allowed to act on urease for 30 minutes) was studied and it was found that when mercury was allowed to act for 30 minutes before BAL was added, the reversal was nil by up to 0.06 μ g. even when BAL was allowed to act on poisoned urease for 30 minutes. BAL obtained from two sources were used in this experiment and practically no difference was found between the two samples of BAL used in this investigation.

Similarly, the reversal effects of BAL was tried when the urease was poisoned with arsenite and it was found that BAL did not produce any reversal effect on the arsenite poisoned urease even when pure BAL was added to the flask containing arsenite and urease.

(i.b.) Mannitol-6-BAL ether:

Similarly, the reversal of mercury poisoning by aqueous solution of mannitol-6-BAL ether was studied on the mercury poisoned urease of 16.VI.47. The results obtained are given in Table 8 and Figure IV.

TABLE 8/



% of reversal of mercury poisoned urease.

Figure IV.

showing the % of reversal with mannitol-6-BAL ether on mercury poisoned urease.

[No face page 33].

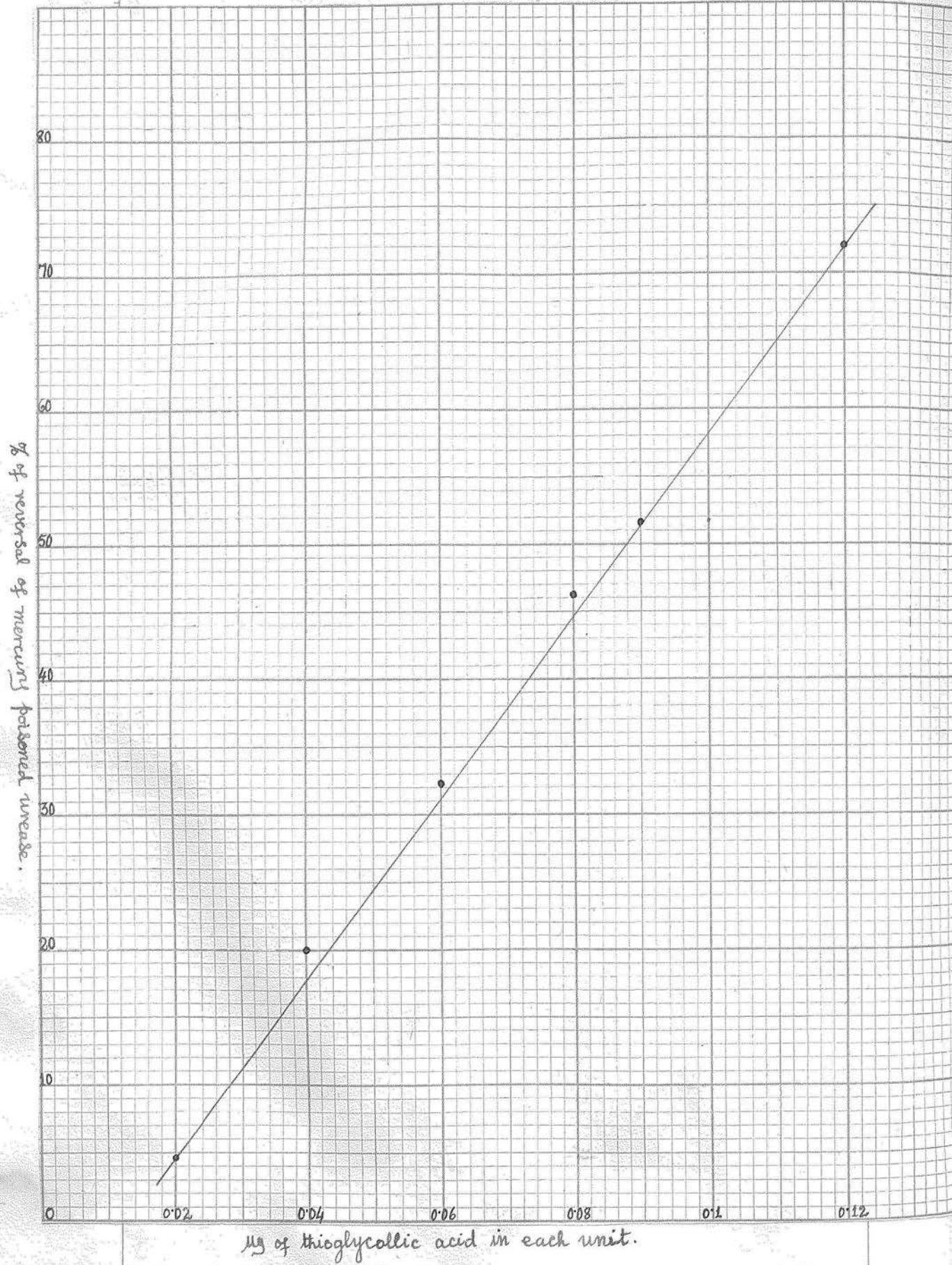
TABLE 8

Reversal effect of mannitol-6-BAL ether on the mercury poisoned urease of

16.VI.47.

µg. of Hg added to each unit	% of inhibition and Range	µg. of mannitol BAL ether added to each unit.					
		0.0086	0.0172	0.0258	0.043	0.0602	0.0688
0.3	78.3 (78.6 to 80)	1.4%	5.1%	8.1%	16%	25%	29.2%
							35.9%

Here also the % of reversal of poisoned urease was proportional to the amount of mannitol-6-BAL ether present in the range of 0.0086 to 0.086 µg.



µg of thioglycollic acid in each unit.

Figure V.
Showing the % of reversal with thioglycollic acid on mercury poisoned urease.

(ii.a) Thioglycollic acid:

Aqueous solution of thioglycollic acid was prepared fresh every day before the experiment and the reversal effect with thioglycollic acid was studied in the same way on mercury poisoned urease of 18.VII.47 as with dithiols. The thioglycollic acid (0.05 ml./46 ml. distilled water) gave the pH about 6 to 6.5 and when added to 1:5 dilution of buffered urease gave a pH 7 to 7.5. The experimental observations are given in Table 9 and Figure V.

TABLE 9

Reversal effect of thioglycollic acid on mercury poisoned urease of 18.VII.47.

µg. of Hg added to each unit	% of inhibi- tion and Range	µg. of thioglycollic acid added to each unit.					
		0.02	0.04	0.06	0.08	0.09	0.12
0.25	80 (78 to 81.4)	4.9%	20%	32.2%	46.2%	51.9%	72%

In the case of thioglycollic acid, the % of reversal of mercury poisoned urease was proportional to the amount of thioglycollic acid present in the range/

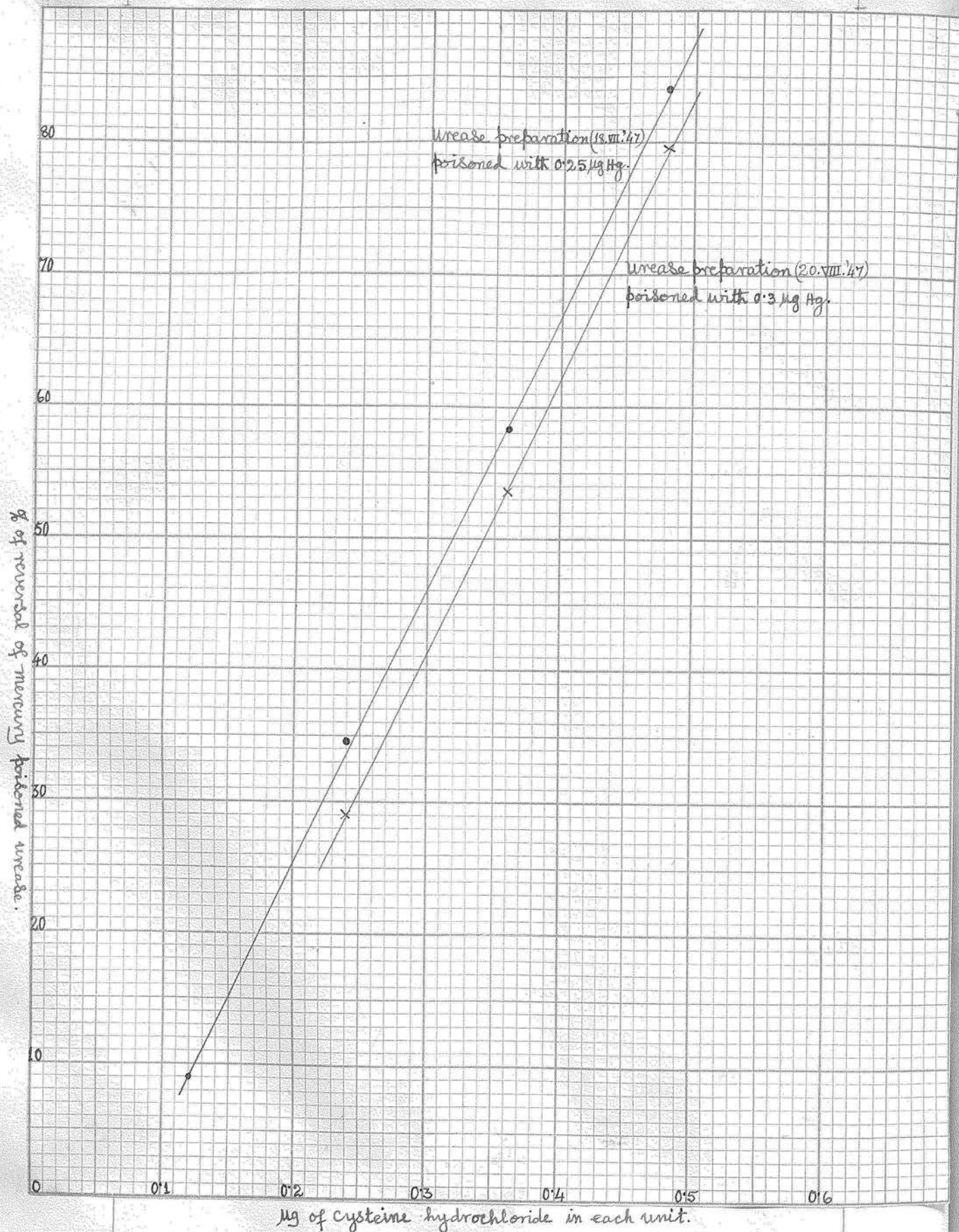


Figure VI.
 showing the % of reversal with cysteine hydrochloride on mercury poisoned urease.

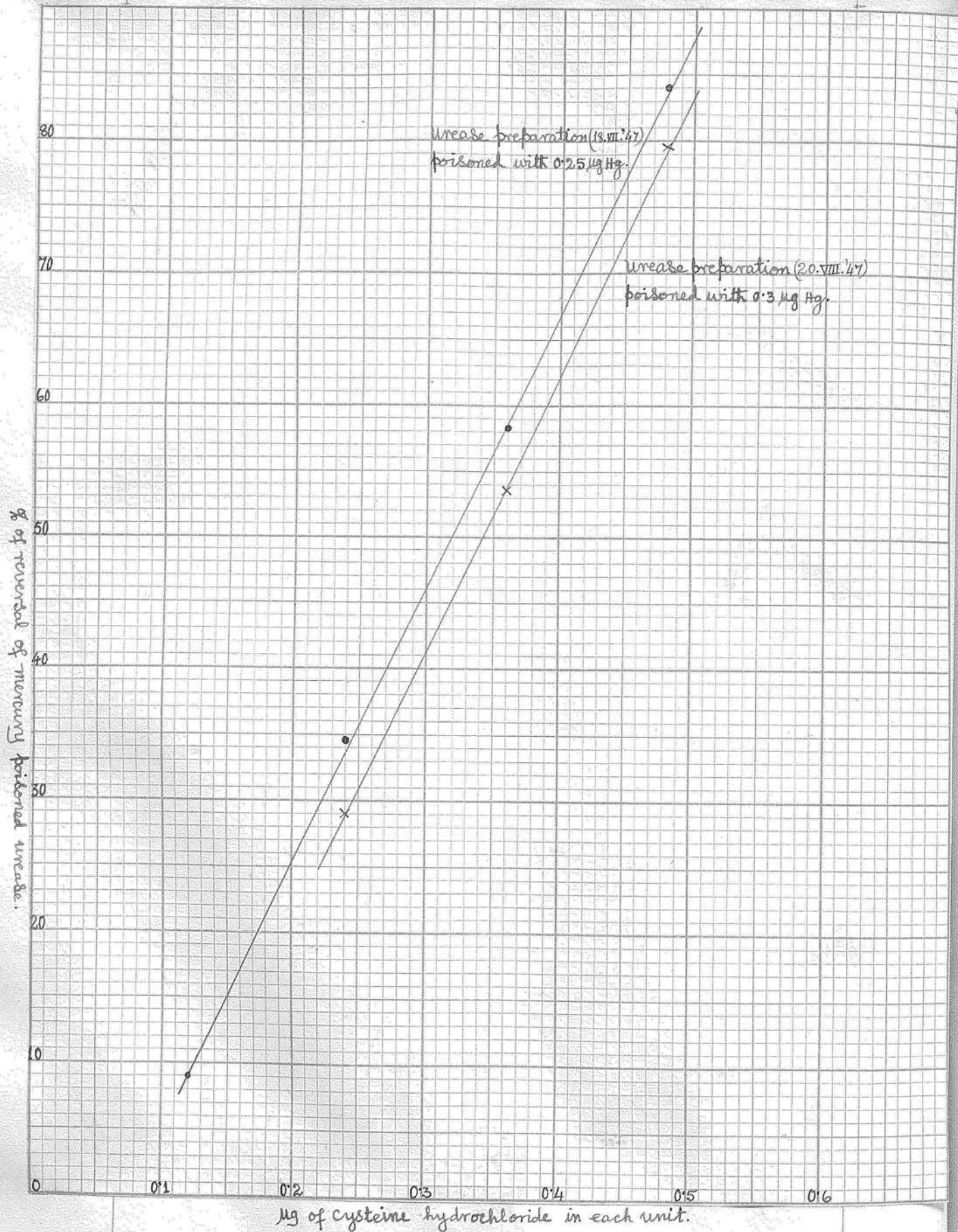


Figure VI.
 showing the % of reversal with cysteine hydrochloride on mercury poisoned urease.

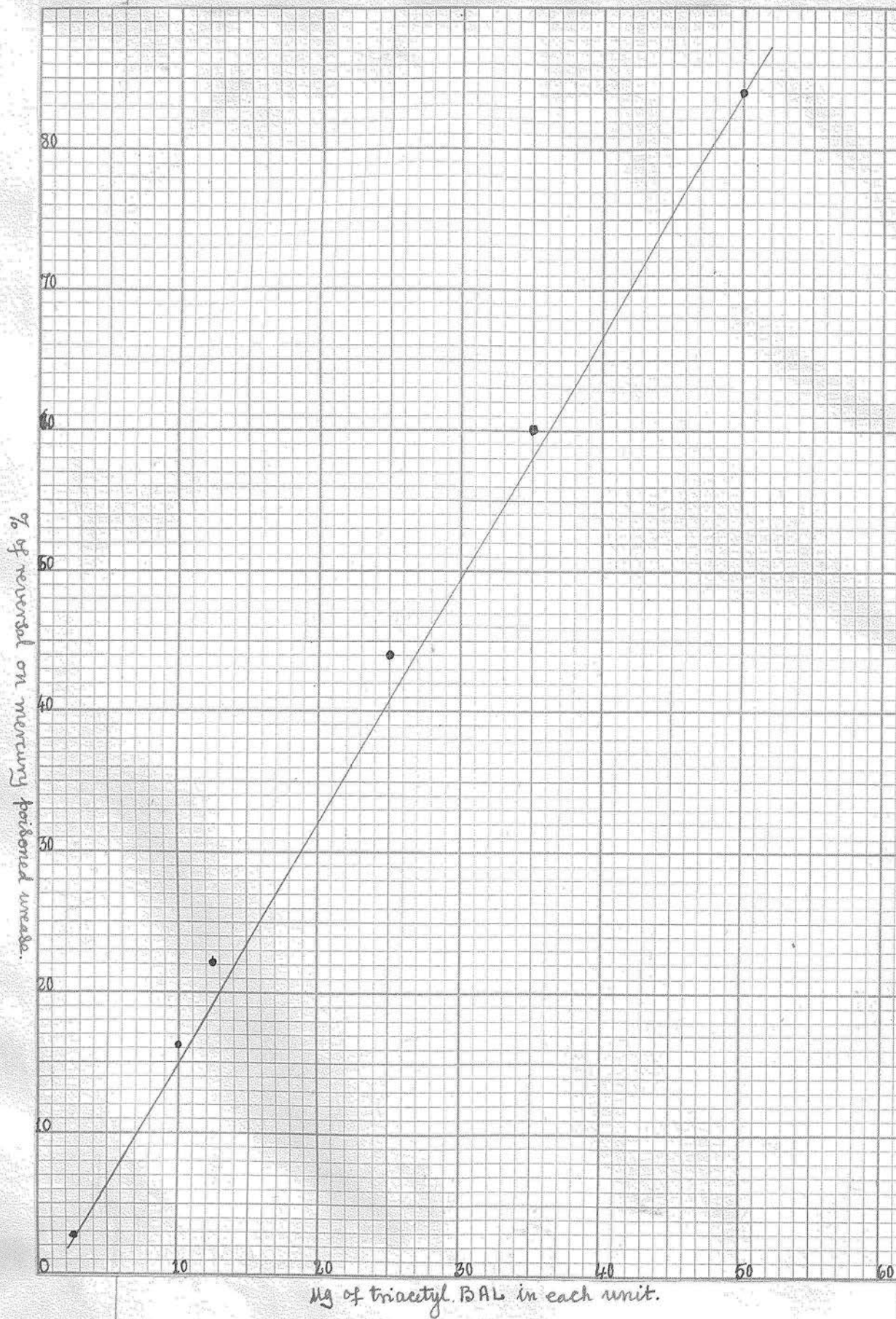


Figure VII.
Showing the % of reversal by triacetyl
BAL on mercury poisoned urease (16.6.47).

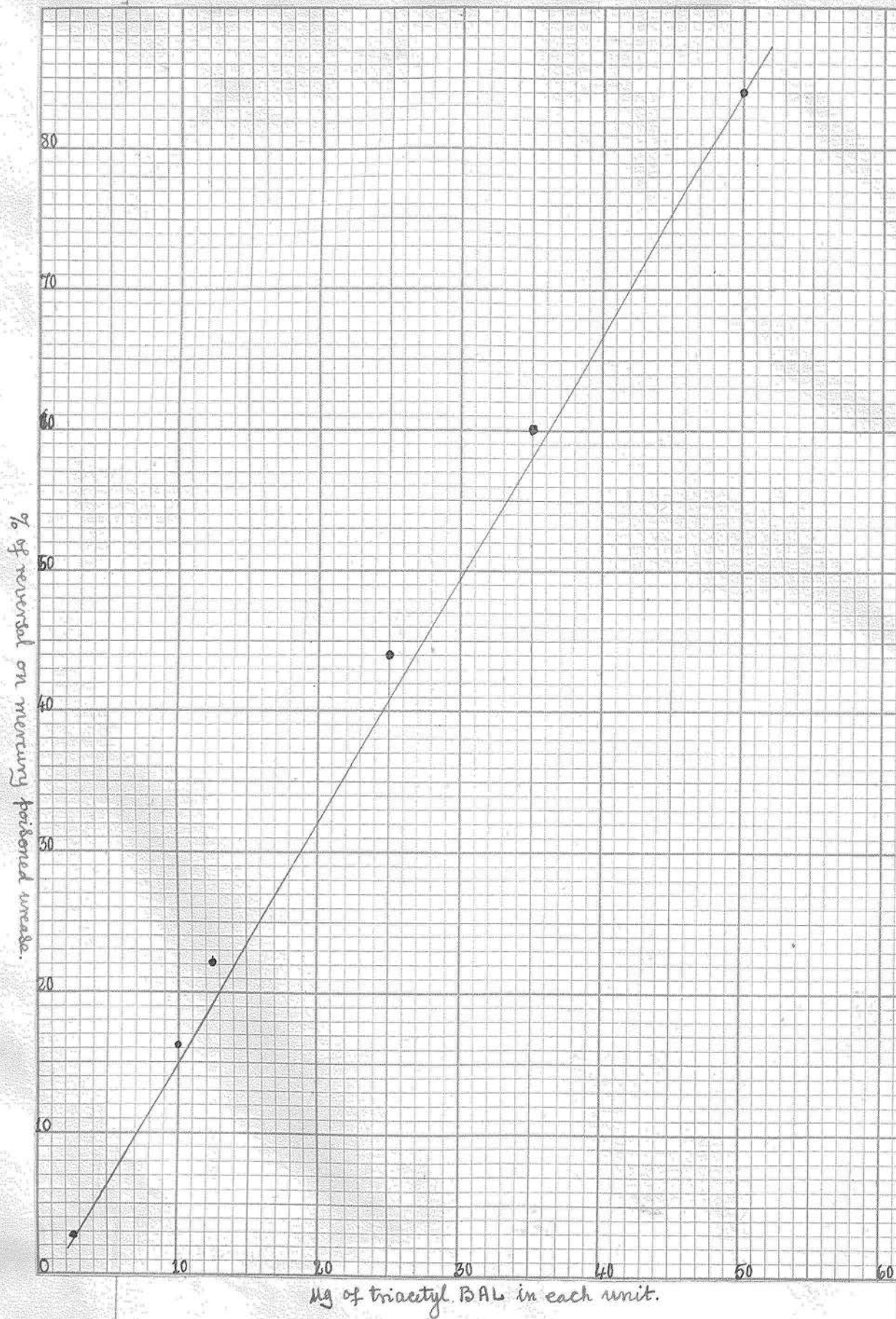


Figure VII.
Showing the % of reversal by triacetyl
BAL on mercury poisoned urease (16.6.47).

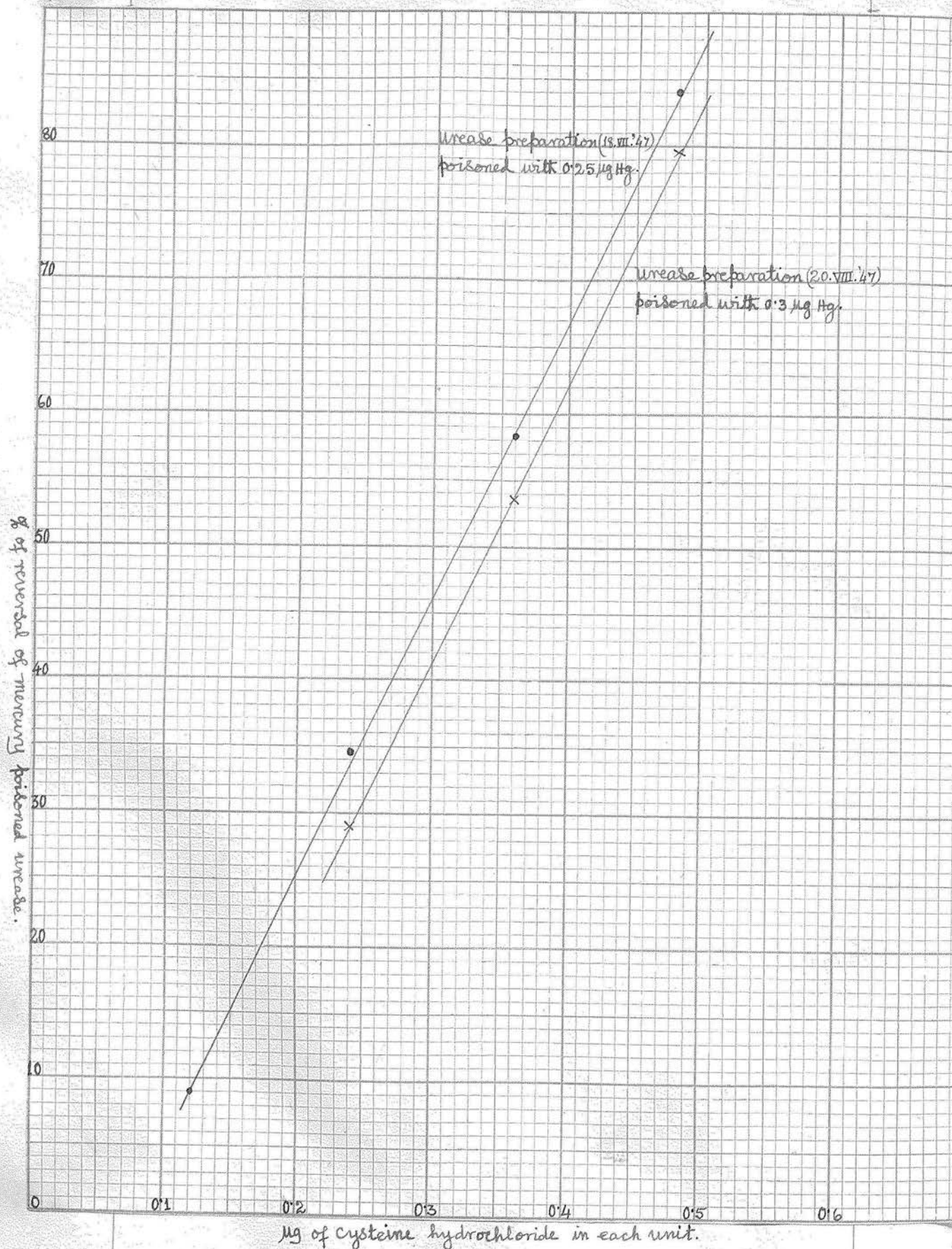


Figure VI.
 showing the % of reversal with cysteine hydrochloride on mercury poisoned urease.

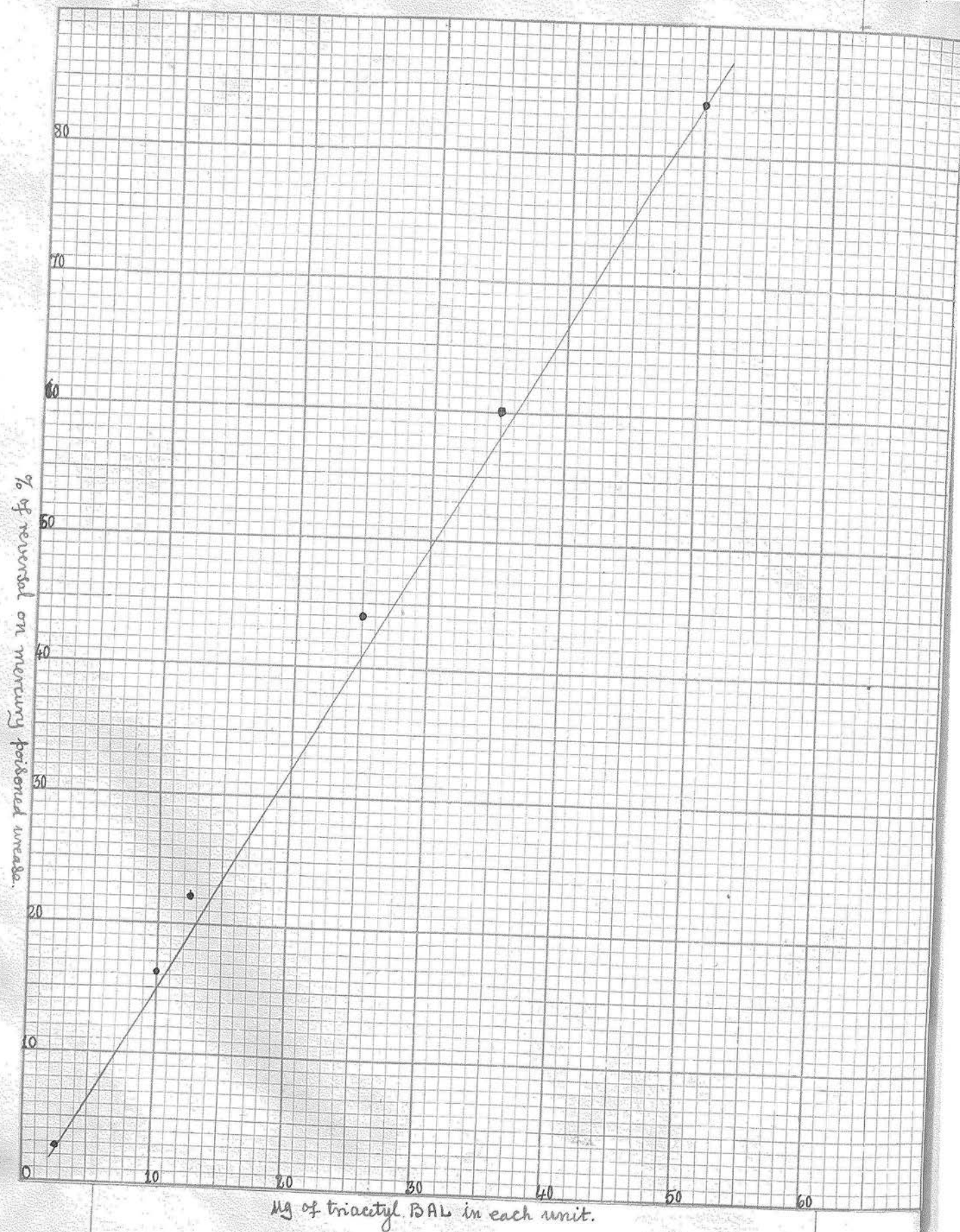


Figure VII.

Showing the % of reversal by triacetyl
BAL on mercury poisoned urease (16.6.47).

proportional to the amount of cysteine hydrochloride present in the range of 0.12 to 0.6 μg .

(iii) Triacetyl BAL:

Reversal effect with aqueous solution of triacetyl BAL in different concentrations and strength was tried on the mercury poisoned urease of 16.VI.47. Table 11 and Figure VII represent the observations.

TABLE 11

Reversal effect of triacetyl BAL on the mercury poisoned urease of 16.VI.47.

μg . of Hg added to each unit	% of inhibition and Range.	μg . of triacetyl BAL added to each unit.					
		2.5	10	12.5	25	35	50
0.3	72.7 (70 to 76.6)	2.9%	16.2%	22%	44%	60%	84%

In this case also the reversal of poisoned urease was proportional to the amount of triacetyl BAL present in the range of 2.5 to 50 μg , but the amounts required to give reversal were of the order of 200 mols of triacetyl BAL to per mol. of mercury. This activity/

activity was probably due to traces of free BAL contained in triacetyl BAL.

(iv) l-cystine:

l-cystine was not soluble in water. So 24 mg. l-cystine was dissolved in one ml. of distilled water and one drop of saturated solution of NaOH and finally the volume was made to 100 ml. with phosphate buffer pH 7.2. The excess of NaOH was not neutralised by Hcl as this process precipitated the cystine from the solution. The pH of this solution was 7.5.

An attempt was made to reverse the inhibited activity of mercury poisoned urease of 20.VIII.47 by adding different amounts of the above solution as in the previous case. The l-cystine solution did not produce any reversal of the mercury poisoned urease when l-cystine was added up to 0.048 mg. or 48 µg. in each unit.

From the foregoing tables and figures the following conclusions could be drawn:

(1) Dithiols - BAL and mannitol-6-BAL ether and monothiols - thioglycollic acid, cysteine hydrochloride were able to reverse the inhibited urease activity which was inhibited up to 70 to 81% of the total with mercury while l-cystine did not produce any/

any reversal up to 48 μ g. Triacetyl BAL, though does not contain any free -SH group proved to be a poor reversal agent, the reversal activity was due to traces of free BAL contamination.

(2) The proportional potency of these dithiols and monothiols which produced reversal of the mercury poisoned urease was as given below, when calculated on 25% reversal of 0.3 μ g. mercury poisoned urease.

0.1 mM BAL = 0.14 mM mannitol-6-BAL ether

= 4 mM thioglycollic acid = 1.16 mM cysteine
hydrochloride

= 44 mM triacetyl BAL,

i.e., BAL is the most potent followed by mannitol-6-BAL ether which is slightly less than BAL. Others in order of merit are cysteine hydrochloride, thioglycollic acid and the triacetyl BAL. Similar result was obtained by Weatherall (1947) with regard to BAL and mannitol-6-BAL ether. She, while studying the toxicity and anti-arsenical activity and some pharmacological properties of certain dithiols and dithiol derivatives found that mannitol-6-BAL ether and mannitol-3-BAL ether are about as effective as BAL in protecting mice against mapharside poisoning.

(3) Even pure BAL could not reverse the arsenite poisoned urease activity.

III. Antimercury activity of rabbits'
plasma after di- and monothiols.

Looney in 1926 first attempted to estimate blood urea by the urease method in a patient who took HgCl_2 by mouth for poisoning. He reported that during bichloride of mercury poisoning, sufficient mercury might accumulate in the blood stream to inhibit the action of urease in the determination of blood urea.

A few years later, Schmidt (1928), while devising a method for the standardization of urease, showed that the sensitivity of the method was greatly decreased in the presence of blood, much more mercury being required to inactivate a definite quantity of urease even when the extra dilution was taken into account.

With a view to working out a method for detection of BAL in blood, first the influence of normal plasma on the mercury poisoned urease was studied by adding 0.1 ml. of normal rabbit's plasma to 5 ml. of 1:5 dilution of urease or of mercury poisoned urease. It was found that normal plasma in these conditions, i.e., one part in 50 potentiated the action of urease by 9 to 14% of the original urease activity while it prevented/

prevented about 82 to 88% of the mercury inhibition of the urease. 1:25 dilution of the original plasma (i.e., a final dilution of 1 in 1250) produced slight or no effect at all on urease and mercury poisoned urease activity (vide infra). So the effect of this dilution of normal plasma of the rabbit was studied on BAL by incubating BAL and diluted plasma for 0 to 30 minutes at room temperature in glass stoppered volumetric flasks. It was noticed that 1 in 1250 dilution of normal plasma of rabbit did not affect the BAL in any way when allowed to act together and the recovery of BAL from such combination was 100%.

(i.a) 2:3 dimercaptopropanol (BAL).

An attempt was made to detect the antimercury effect of rabbits' plasma after intramuscular injection of BAL. For this purpose doses of 50 mg. BAL/kg. (0.4 mM BAL/kg.) dissolved in 0.5 ml. 66% solution v/v of propylene glycol were injected intramuscularly in rabbits. Blood from the ear veins was collected before and 30 minutes after the injection. The blood samples were centrifuged and clear supernatant plasma was separated. The reversal of mercury poisoned urease/

urease was studied using plasma diluted in 1:1250 various experiments were carried out in which the reagents were added in different orders and at different intervals of time. In some experiments mercury was added to the urease and the plasma was added one minute later. If 0.5 ml. of this mixture was added to the urea in the Conway unit 2 minutes later, the BAL in the plasma had no effect, but if it was left for 15 to 60 minutes before it was added to the urea, the BAL was effective. In these conditions the disinhibition thus proceeds much more slowly than when pure BAL was added to poisoned urease. An experiment was therefore done in which BAL and normal plasma were mixed and left for 2, 10 and 15 minutes before being added to the poisoned urease. In these conditions the disinhibition was rapid as with pure BAL. The plasma taken from the rabbit which had received BAL does not therefore consist of simple mixture of BAL and plasma since it acts more slowly.

such or in dilutions of 1:5, 1:10 and 1:100 did not affect either the mercury inhibition or the reversal by BAL in vitro nor did doses of 0.5 ml. injected intramuscularly alter the activity of plasma.

During subsequent experiments the rabbits were injected intramuscularly with 0.4 mM BAL/kg. in 0.5 ml. propylene glycol. Blood from ear veins was collected before and 30, 60, 90, 120 and 180 minutes after the injection. Similar tests for estimating the anti-mercury activity present in all the samples of plasma were performed. As in none of the cases did the diluted sample of plasma obtained before the injection had any antimercury activity, while all the samples of plasma obtained at different time intervals after the injection produced marked reduction in the mercury inhibition, it seemed likely that the marked reversal was due to the presence of BAL or derivatives of it. Assuming the activity to be due to BAL itself, the amount actually present in different samples of plasma after the injection of BAL was deduced from the calibration curve for BAL (Figure III) as shown below.

TABLE 12/

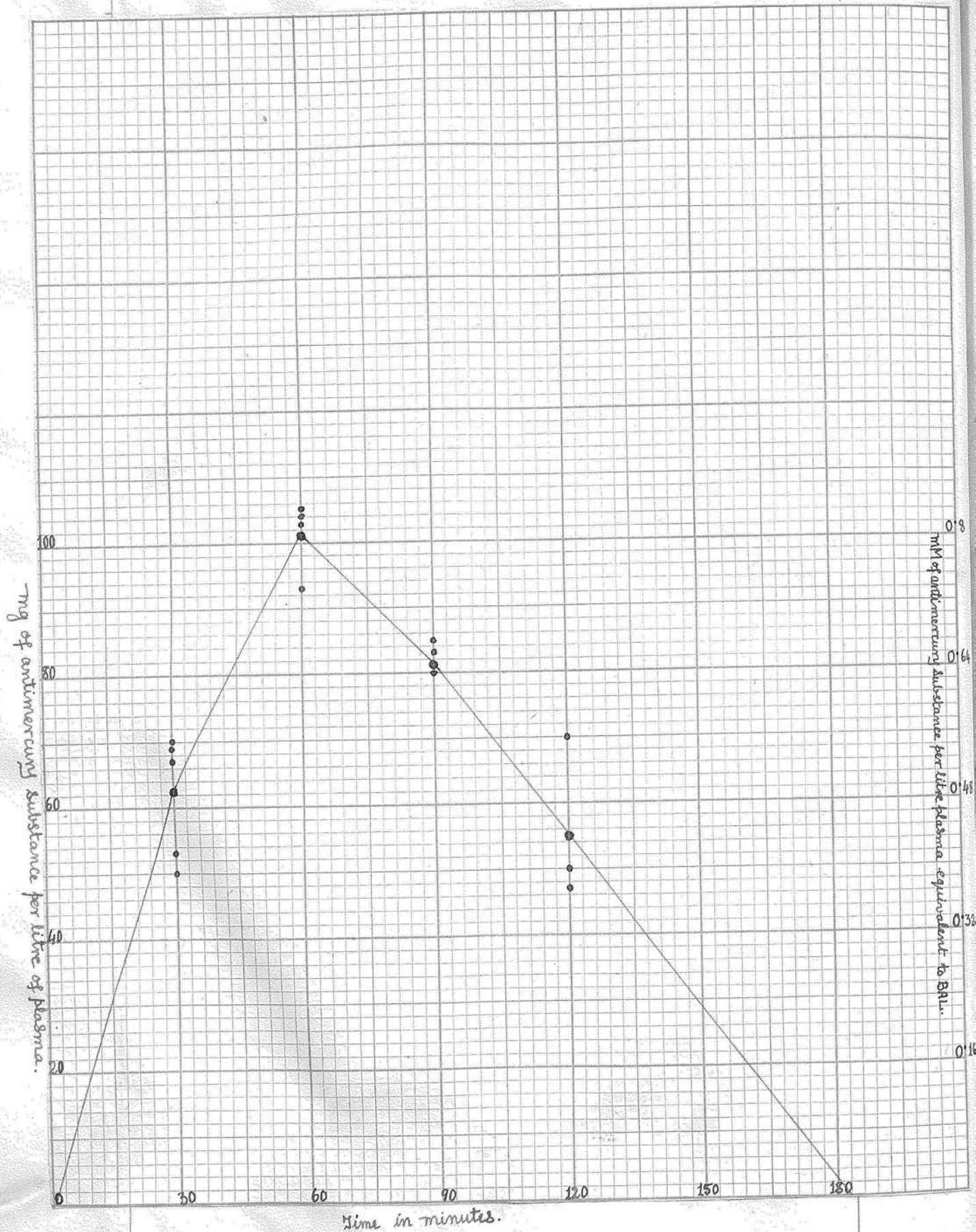


Figure VIII.
 Showing the antimercury substance
 per litre plasma after 0.4 mM or 50 mg
 BAL/kg.

[To face page 44].

TABLE 12

Showing the % of reversal and the corresponding quantity of BAL or its derivative in mg./litre in plasma after 50 mg. BAL/kg. or 0.4 mM/kg. was injected intramuscularly in rabbits.

30 minutes		60 minutes		90 minutes		120 minutes		180 minutes	
% of reversal	Corresponding mg./litre	% of reversal	Corresponding mg./litre	% of reversal	Corresponding mg./litre	% of reversal	Corresponding mg./litre	% of reversal	Corresponding mg./litre
14.6 ⁺ -2.7	62.5 ⁺ ±10.7	25.4 ⁺ ±1.4	101.5 ⁺ ±5.8	20.6 ⁺ ±0.7	81.5 ⁺ ±2.5	12.9 ⁺ ±3.5	55 ⁺ ±12.5	2.1	Just detectable

Table 12 and Figure VIII show that antimercury substance, presumably BAL or derivatives of BAL, was present in gradually increasing amount in samples of plasma obtained 30 and 60 minutes after the injection (62.5 mg./litre at 30 minutes and 101.5 mg./litre at 60 minutes) after which there was reduction of antimercury activity in plasma until it was just detectable at 3 hours.

(i.b) Mannitol-6-BAL-ether:

Aqueous solutions of mannitol-6-BAL ether 115.2 mg./kg. (0.4mM/kg.) were injected intramuscularly into rabbits. This time the drug was not mixed or diluted with propylene glycol as the volume of the drug in aqueous solution was always more than 0.5 ml. Samples of blood before and 30, 60, and 120 minutes after the injection were collected and were examined for mannitol-6-BAL ether or for its derivatives in 1:25 dilution as in the case of BAL. The amount present in plasma was deduced from the mannitol-6-BAL ether calibration curve (Figure IV) which has been given in Table 13 and Figure IX.

TABLE 13/

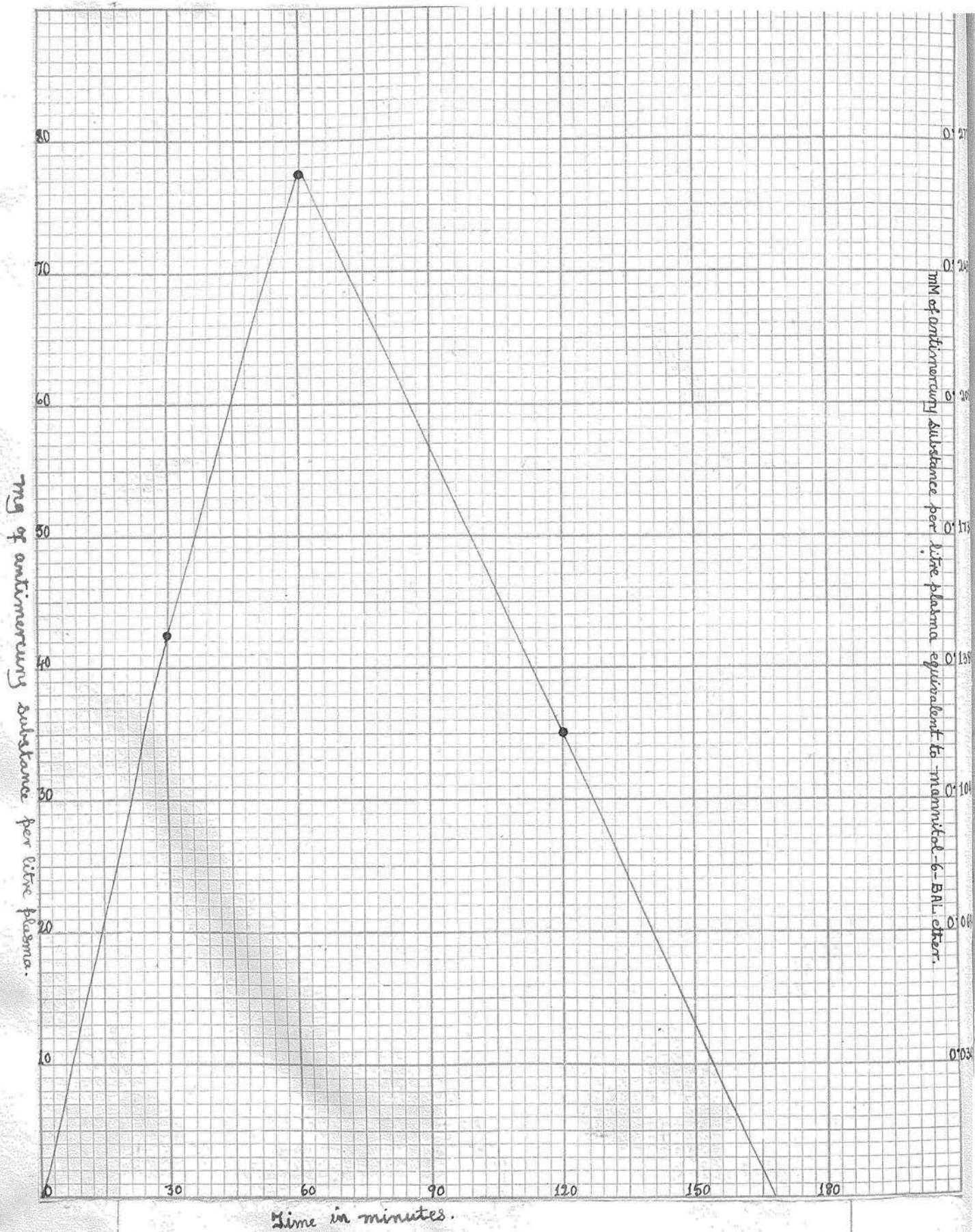


Figure IX.
 Showing the antimercuric substance
 per litre plasma after 0.4 mM or 115.2 mg
 mannitol-6-BAL ether/kg.

[To face page 46].

TABLE 13

Showing the % of reversal and the corresponding quantity of mannitol-6-BAL ether or its derivative in mg./litre present in plasma after 0.4 mM mannitol-6-BAL ether/kg. was injected intramuscularly in rabbits.

Red figures represent the number of observations in each case.

30 minutes		60 minutes		120 minutes	
% of reversal	Corresponding mg/litre	% of reversal	Corresponding mg/litre	% of reversal	Corresponding mg/litre
5	42.5 (2)	11.3	77.5 (3)	3.8	35 (2)

Assuming that the antimercury substance, present in the samples of plasma obtained after the injection of mannitol-6-BAL ether, to be either the mannitol-6-BAL ether or some derivative of approximately equal antimercury activity, the amount varies according to the time interval after the injection at which the/

the blood was collected. Thus 30 minute sample plasma shows 42.5 mg./litre rising up to 77.5 mg./litre in 60 minutes and then declining to 35 mg./litre in 120 minutes.

(ii.a) Thioglycollic acid:

As this solution was highly acidic, 36.8 mg./kg. of thioglycollic acid was diluted in 0.5 ml. 66% propylene glycol and neutralised with NaOH, before it was injected intramuscularly in the rabbits. Samples of blood before and 30, 60 and 120 minutes after the injection were collected. Studies for detection of antimercury substance in the plasma were made with 1:25 dilution of plasma as on previous occasions. On the assumption that the antimercury substance present in different samples of plasma collected after the injection was either thioglycollic acid or some equally active derivative, the equivalent amount was deduced from the thioglycollic acid calibration curve (Figure V). The results obtained are shown below.

TABLE 14/

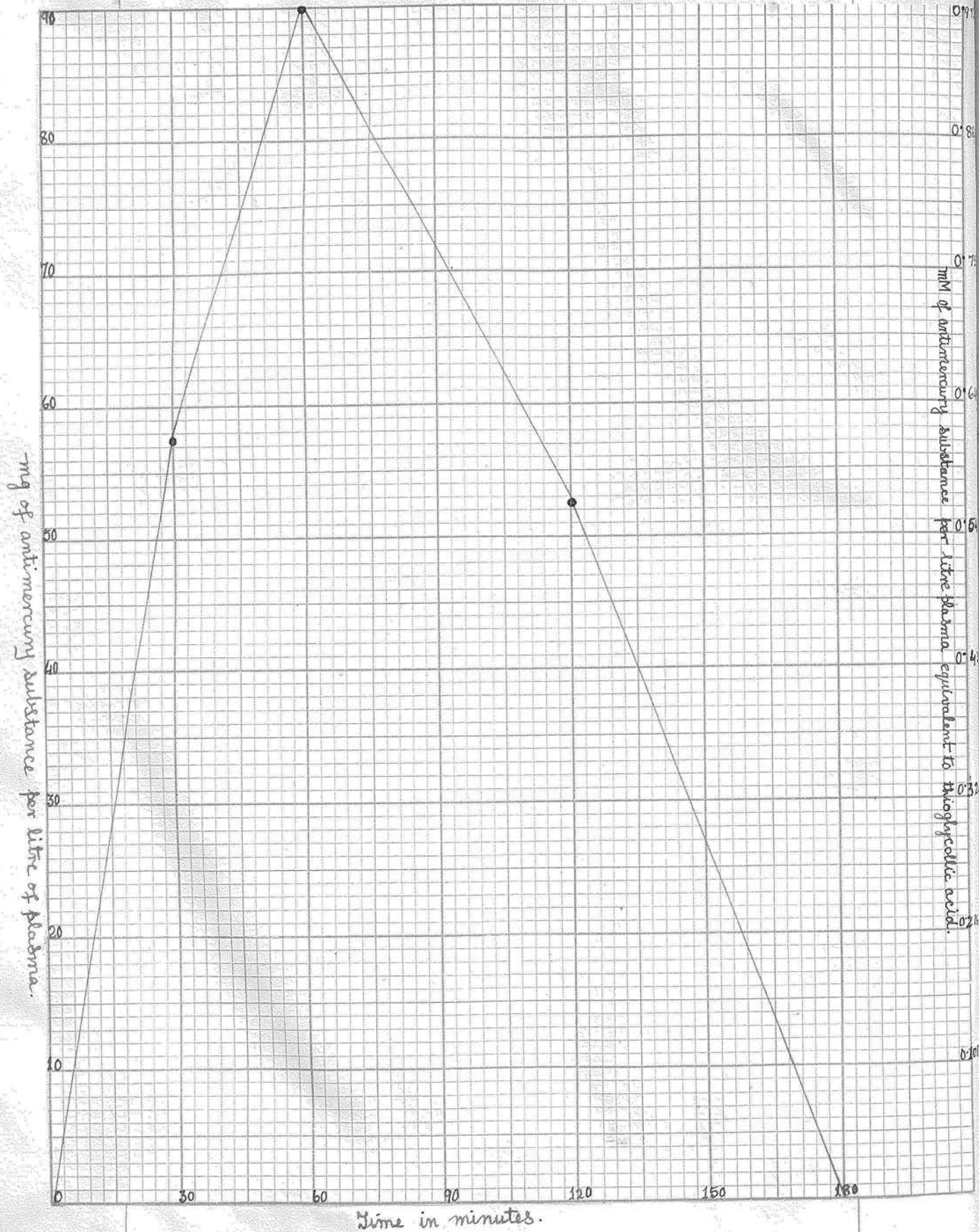


Figure 8.
 showing the antimercurey substance
 per litre plasma after 0.4 mM or 36.8
 mg thioglycolic acid/kg.

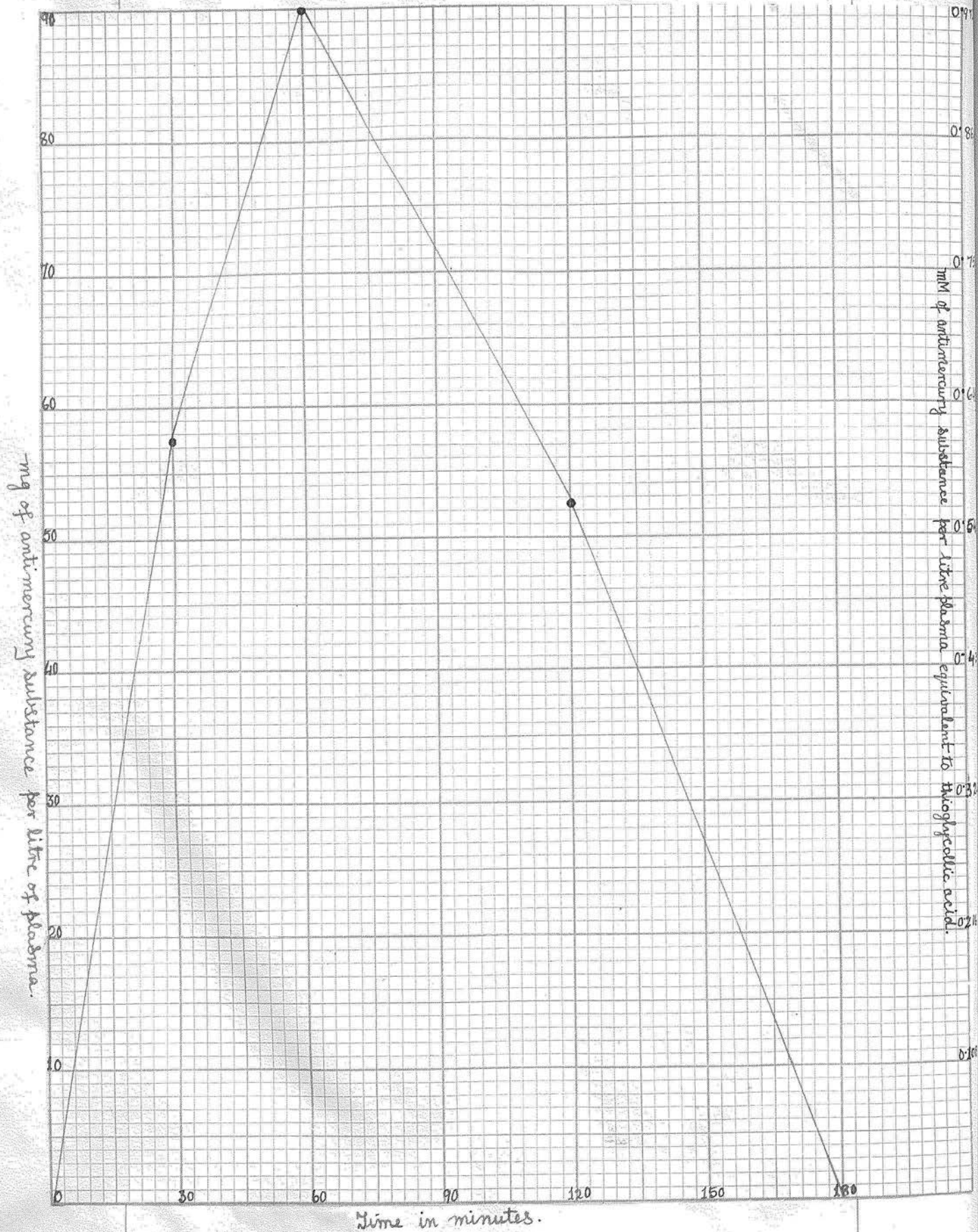


Figure 8.

showing the antimercurey substance per litre plasma after 0.4 mM or 36.8 mg thioglycolic acid/kg.

mM equivalent of thiols.

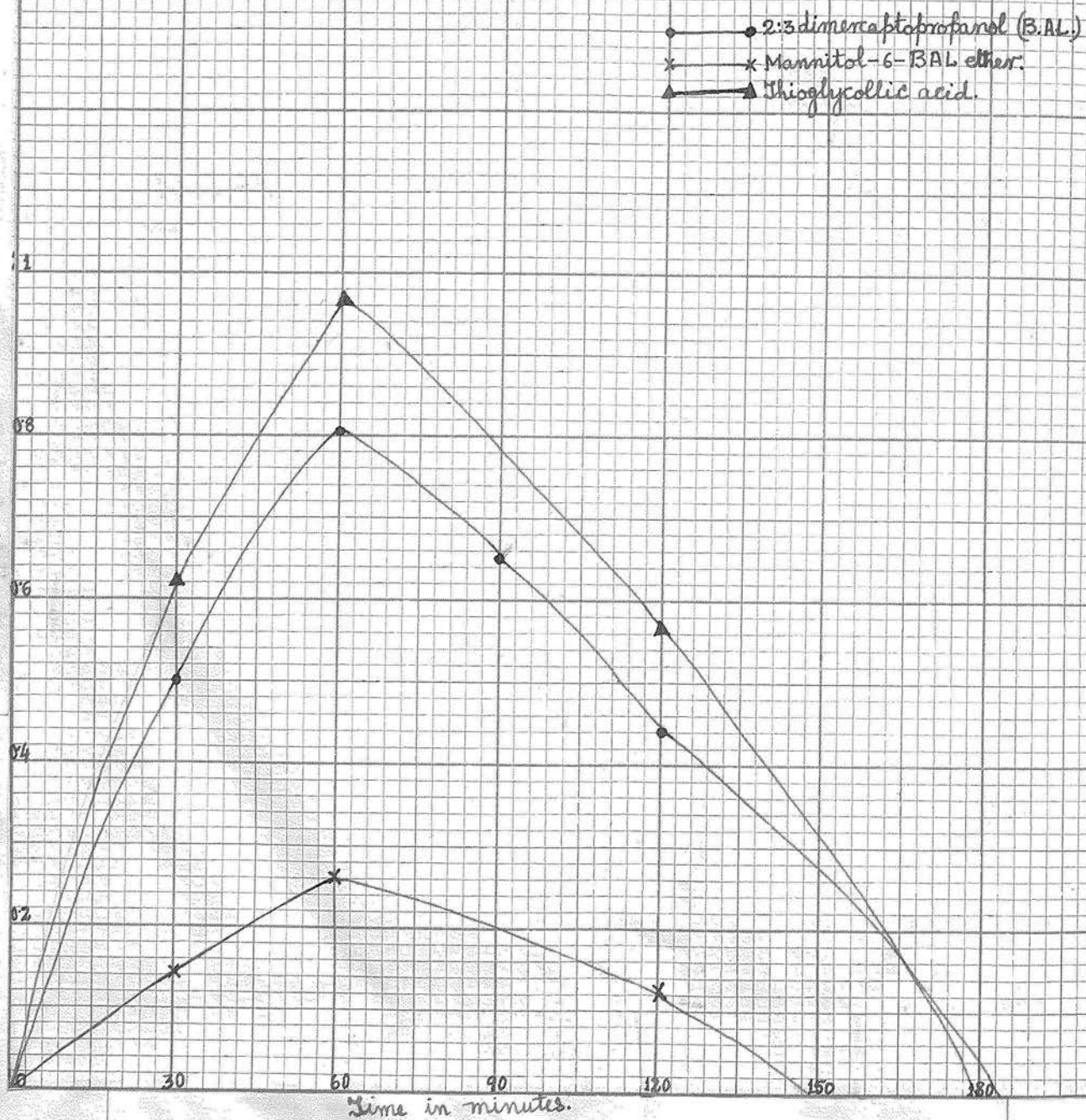


Figure XI

Showing the Concentration of various thiols per litre plasma after intramuscular administration of 0.4 mM thiols/kg.

before and $\frac{1}{2}$, 1, 2, 4 and 6 hours after injection, was tested. None of the samples, collected either before or after the injection, showed any reversal of mercury poisoned urease, so it was concluded that the hydrolysis of triacetyl BAL is either too small and slow or not at all in such experiments.

Taking into consideration all the thiols injected intramuscularly in doses of 0.4 mM/kg. (i.e., BAL 50 mg./kg; mannitol-6-BAL ether 115.2 mg/kg; thioglycollic acid 36.8 mg/kg; cysteine hydrochloride 48 mg./kg. and triacetyl BAL 100 mg./kg), a graph, Figure X1, has been plotted to show the relative rates of absorption and of their presence in the plasma. From this figure it is apparent that of all the thiols 2:3 dimercaptopropanol (BAL) was present longest in the plasma as its presence was just detectable even 3 hours after the injection (2.1% reversal). Although thioglycollic acid reached the highest molar concentration in plasma, its effect was less than that of the BAL because it is less active against mercury. Mannitol-6-BAL ether comes last. Cysteine hydrochloride when/



when injected intramuscularly in doses 0.4 mM/kg. in rabbits could just be detected in the sample of plasma taken 60 minutes after injection. Triacetyl BAL could not be detected at all in any sample of plasma after 0.4 mM/kg. intramuscularly injected in rabbits though an attempt to detect it was made up to 6 hours. From all these it could be concluded that 2:3 dimercaptopropanol (BAL) was the most readily absorbable one from among the di- and monothiols studied during this investigation.

IV. Antimercury activity of plasma.

As indicated in the previous chapter, it has been observed that normal rabbits' plasma potentiated the activity of urease and at the same time prevented the poisoning of urease by mercury. It was thought justifiable to see the effect of repeated small doses of mercury on rabbits' plasma. With this object in mind the following experiments were performed.

Two rabbits were injected each with 1 mg./kg. of mercury intramuscularly every day till they showed toxic manifestations either in the form of diarrhoea or haematuria. The antimercury effect of plasma was estimated daily. One of these rabbits developed diarrhoea on the 6th day after the start of injection, i.e., after getting a total of 5 mg. Hg/kg. and the injection was stopped. At this stage, 0.1 ml. of undiluted blood plasma when added to 1:5 of urease did not show any augmentation of the urease activity (cf. normal plasma which showed 13.9% potentiation). The diarrhoea stopped in a day without any treatment. The behaviour of plasma was tested from time to time till/

till after a month of stopping the mercury injection. The plasma 0.1 ml. undiluted did not show any recovery of the lost potentiation activity at this stage, so BAL 20 mg./kg. with 0.5 ml. propylene glycol was injected three times every day for three days. On the 4th day again the behaviour of plasma was tested and no difference was noted even after the treatment with BAL. Lastly, the plasma was again tested six weeks after the treatment with BAL when it was found that the plasma had regained its normal potentiation activity for urease.

The other rabbit did not show any toxic manifestation on the 6th day of Hg injection, but certainly the plasma had lost its potentiation activity as in previous case, so to see whether by pushing in more mercury any further change could be brought about in plasma, the injection of mercury every day was continued till on the 9th day, i.e., after a total of 9 mg. Hg/kg, the rabbit developed haematuria and paresis of all four limbs, more marked in hind limbs. The mercury injections were stopped. The plasma at this stage did not show any further change as compared to the 6th day, i.e., the abolition of antimercury effect of plasma on the 9th day was the same as it was on the 6th day. At this/

this stage an attempt was made to treat this rabbit with BAL 20 mg./kg. intramuscularly three times a day, but after only one injection of BAL the rabbit died and on post-mortem the following changes were observed.

(1) Heart:- minute subpericardial haemorrhages were detected. There was no effusion in the pericardial sac. The heart was found in a state of systole and there was no blood or blood clot in any of the heart chambers.

(2) Kidneys:- the most marked changes were detected in the kidneys. These were markedly enlarged and evidences of subcapsular haemorrhages were found. The capsule stripped very easily. On cutting, the medullary zone was found to be well marked out from cortex and blood dripped from the cut surfaces.

(3) Bladder:- the bladder was full and distended with haemorrhagic and smoky urine. The bladder was found congested and showed some small haemorrhages.

(4) Central nervous system:- petechial and diffuse haemorrhages were detected in the spinal cord/

cord in the mid-thoracic, lower thoracic and in upper lumbar regions. The brain did not show any evidence of inflammation or haemorrhage.

(5) In subcutaneous area also petechial haemorrhages were detected but these were not so marked. Besides these post-mortem findings other viscera and organs of the rabbit were normal. The changes found are typical of subacute mercury poisoning. (Waife and Pratt, 1946 and Smith, 1943).

V. Antimercury activity of plasma after
dithiol in liver damaged rabbits.

Cameron, Burgess and Trenwith (1947) reported that BAL was more toxic when the liver was damaged by carbon tetrachloride. They observed an increased mortality (90%) in rabbits with liver damage during 24 hours after administration of 80 mg./kg. BAL as compared with normal group (53%). A difference was present too in their experiment with rats. An even more striking feature of the hepatic damage group was the development of toxic symptoms in the animals receiving low doses of BAL. The normal controls with the same doses of BAL showed no such symptoms. Cameron et al could not detect any structural damage in the liver, attributable to BAL, but on the large percentage of death after injection of BAL in liver damaged cases, they conjectured that the normal liver might be playing a part in detoxication of BAL.

With a view to finding out whether healthy liver plays a part in detoxication of BAL, experiments were planned to estimate the quantity of BAL in rabbits plasma after 20 mg/kg. in normal and in experimentally liver damaged rabbits.

Three/

Three apparently normal healthy rabbits weighing 1.75 to 2.1 kg. were injected each with a 20 mg. BAL/kg. intramuscularly as before. Blood samples before and 30, 60 and 120 minutes after injection were collected and plasma was separated. Estimations of antimercury activity of plasma were done as in previous experiments and the results obtained are given in Table 15.

One set of four rabbits were injected subcutaneously with purified carbon tetrachloride each with a single dose of ml./kg. according to Cameron and Karunaratne (1936). Three of these four rabbits died within two days of Ccl_4 injection. On post-mortem examination all showed severe degree of fatty infiltration and degeneration of liver. The one remaining was injected with 20 mg. BAL/kg. intramuscularly on the fourth day after Ccl_4 injection. The samples of blood before and 30, 60 and 120 minutes after the injection were collected for estimation of antimercury activity present in plasma.

One rabbit was injected subcutaneously with 0.8 ml./kg. purified Ccl_4 and again 20 mg. BAL/kg. was injected intramuscularly in this rabbit on the fourth day after the Ccl_4 injection. Estimations were/

were done for the antimercury activity present in plasma as before. The results obtained are given in Table 15.

TABLE 15

Showing the antimercury activity present in the samples of plasma obtained before and 30, 60 and 120 minutes after the intramuscular injection of 20 mg./BAL/kg. in normal and experimentally liver damaged rabbits.

Condition of rabbits.	Weight of rabbits	% of inhibition of urease with 0.3µg.Hg/unit:-		% of reversal in different samples of plasma⊗		
		with-out plasma	with pre-injection plasma	30 minutes	60 minutes	120 minutes
Normal	(1.75kg.	76.9	73.6	4	1.4	nil
	(1.95kg.	78.7	75.3	5.1	2	nil
	(2.1kg.	76.6	76	4.8	2	nil
Liver damaged	(1.75kg.	71	68.3	4.8	24.3	nil
	(1.5kg.	76.9	74.1	4.8	17.3	nil

⊗ Collected after the injection of BAL.

From Table 15 it is evident that before BAL was given/

given the plasma of normal healthy and of the liver damaged rabbits behaved in a similar way, i.e., practically there was no difference in the inhibition produced by the samples of plasma collected before the injection of BAL of the normal healthy rabbits and of the liver damaged ones (cf the difference of inhibition of urease produced by 0.3 $\mu\text{g}\cdot\text{Hg}/\text{unit}$ with or without the diluted plasma in both the sets ranged between 0.6 to 3.3%). In liver damaged rabbits the samples of plasma obtained 30 and 60 minutes after the injection of BAL showed much more antimercury activity, i.e., 4.8 to 24.3% reversal in comparison to antimercury activity shown by normal healthy rabbits' plasma under the same conditions which showed 1.4 to 5.1% reversal with samples of plasma obtained 30 and 60 minutes after BAL injection. In the liver damaged rabbits the 30 minutes samples practically showed the same range of antimercury activity as in normal case, but it was 60 minutes samples which showed the contrast difference - 1.4 to 2% reversal in normal rabbits in comparison to 17.3 to 24.3% reversal in liver damaged ones. This suggests that in normal rabbits as the concentration of BAL was rising/

rising in blood with the lapse of time and was being brought to liver cells, it is getting destroyed there gradually up to certain limits.

VI. Antimercury activity of rabbits'

urine after dithiol.

In Oxford in 1941 Stocken and Thompson demonstrated that shortly after the injection of BAL into rabbits and rats the urine of the animals developed a strong nitroprusside reaction for thiol groups accompanied by a large increase in the iodine titre of the urine. The nitroprusside reaction, which is ordinarily negative, again disappeared and the iodine titre fell to its previous value within a few hours of the injection.

In order to obtain more precise information regarding the nature of the urinary thiols Spray, Stocken and Thompson (1947) attempted to estimate quantitatively the thiols present in the urine of rabbits after the intramuscular injection of BAL. They employed the Cobalt colour reaction for estimation of BAL as described by Calvery (1943) and Spray (1947) and the iodine titration method. They could recover only 10 to 30% of the injected BAL by the iodine titration method in 5 to 8 hours after injection of 40 to 50 mg. BAL/kg. The Cobalt method yielded only up to 10% of the 40 to 50 mg. BAL/kg. injected into the rabbits. They later tried extraction/

extraction of thiols from the urine by Benzene or by precipitation of the Thallium complex. They observed that only 20% of the thiols passes into the benzene layer in the first extraction, instead of 50% as is found with BAL added to urine. So they employed the thallium sulphate method of extraction of BAL from the urine and have shown that rabbits excrete in the urine a considerable quantity of thiols, representing some 20% of the thiol content of the injected BAL, and that a considerable proportion of this excreted thiol is present as dithiol related to BAL.

In the present experiment, first samples of urine from normal rabbits, collected in metabolism cages were tried for their effects on urease and mercury poisoned urease. These urines gave very variable results probably due to contamination with the metal of the cages, so catheter specimens of rabbits' urine were employed for further investigations. Catheter specimens of rabbits' urine were tested for interference on urease and on mercury poisoned urease in undiluted and in various dilutions (1:10 to 1:100). As the urine obtained from the same rabbit on different occasions gave different results even at the same dilution/

dilution, 1:100 dilution (i.e., the final dilution in the reaction mixture 1:5000) at which none of the samples of urine showed any interference with urease or mercury poisoned urease, was employed in subsequent investigations.

The influence of this dilution of rabbit's urine was tested on BAL in vitro experiment. 0.1 ml. urine and 50 µg.BAL (0.05 ml. of 0.04 ml./50 ml. fresh solution) were kept in glass stoppered 10 ml. volumetric flasks for $\frac{1}{2}$, 1, 2 and 4 hours at room temperature. The amount of recovery of BAL in each case was estimated by adding 0.1 ml. (1:100) of one of these incubated urine BAL mixtures as in the previous occasions. The results obtained are given in Table 16.

TABLE 16/

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TABLE 16/

TABLE 16

Showing the amount of recovery of BAL from urine BAL mixture when incubated for $\frac{1}{2}$, 1, 2 and 4 hours at room temperature.

Without urine and with 0.05 μ g. BAL/ unit.		Urine + 0.05 μ g. BAL/unit incu- bated for $\frac{1}{2}$ hour.		Urine + 0.05 μ g. BAL/unit incu- bated for 1 hour.		Urine + 0.05 μ g. BAL/unit incu- bated for 2 hours.		Urine+0.05 μ g. BAL/unit incu- bated for 4 hours.	
% of reversal	% of recovery	% of reversal	% of recovery	% of reversal	% of recovery	% of reversal	% of recovery	% of reversal	% of recovery
62.6	100	62	92	57.1	84	18.6	26	-	nil
69.3	100	69.2	100	54	80	-	-	-	nil
63.5	100	61.1	90	56.3	82	18.0	26	-	nil
65.2	100	64.1	95	55.7	83	18.3	26	-	nil

Table 16 shows that incubation of BAL with urine in vitro experiments did affect the recovery of BAL from the urine-BAL mixture as shown above and it was only possible to recover about 100% BAL from such mixtures within 30 minutes, beyond which the recovery of BAL diminished as the incubation time was prolonged until after 4 hours no recovery was possible. So it seems probable that by keeping BAL up to 50 µg. with 0.1 ml. rabbits' urine for 4 hours, either BAL gets destroyed or it gets into some stable combination from which its recovery is not possible under ordinary conditions.

For studying the presence of antimercury activity in urine after administration of BAL two sets of experiments were planned. In one set the urine of the rabbits was collected from a catheter and 50 mg. BAL/kg. with 0.5 ml. 66% propylene glycol was injected intramuscularly. In the other set, after catheterisation, the rabbits were given 25 ml. water/kg. by stomach tube and the same amount of BAL was injected intramuscularly. From both the sets of rabbits catheter specimens of urine were collected 2, 4 and 7 hours after the injection of BAL. Estimations for antimercury activity present in the samples/

samples of urine obtained before and after the injection of BAL were made with the final dilution of urine 1:5000 as in the previous case. As none of the samples obtained before the injection of BAL showed any antimercury activity while all the samples of urine obtained in both the sets of rabbits showed varying amounts of antimercury activity; So the total amount of antimercury activity calculated in terms of equivalent amount of BAL was deduced from the calibration curve for BAL (Figure III).

The results obtained are given in Table 17.

TABLE 17/

TABLE 17.

Showing the amount of antimercury activity of urine in rabbits after 50 mg. BAL/kg. was injected intramuscularly with or without water diuresis.

Rabbits	Wt. in kg.	BAL inj- ected in mg.	2 hours		4 hours		7 hours		Total amount excre- ted.	% of total amount admini- stered.
			volume	mg. of BAL	volume	mg. of BAL	volume	mg. of BAL		
48	1.6	80	3.6ml.	2.9	12.2ml	1.8	-	-	4.7mg.	5.8
302	2	100	0.8ml.	3.4	1.3ml	1.9	-	-	5.2mg.	5.2
After 25 ml. water/kg. by stomach tube in each rabbit:-										
302	1.7	85	7.6ml.	4.33	3.9ml.	1.09	-	-	5.42mg	6.3
Gray	1.95	97.25	11ml.	4.6	5.2ml.	1.48	3	gave 5.1% reversal	5.98mg	6.1

From the above table it appears that total % of antimercury activity shown by the urine after intramuscular administration of BAL is practically the same in both sets of experiments (about 6% of the total amount of BAL injected) and the diuresis produced by water introduced by stomach tube did not substantially affect the total % of excretion of antimercury substance present in the form of BAL or its derivative though the total volume of urine in both the cases were different.

In spite of the inactivating effect of urine on BAL as shown by in vitro experiment, it was possible to detect the presence of antimercury substances in sample of urine even 7 hours after the injection of BAL.

VII. Antimercury activity of urine
after dithiol in kidney damaged rabbits.

Cameron, Burgess and Trenwith (1947) studied the possibility of toxic effects from 2:3 dimercapto-propanol in conditions of impaired renal function. They showed that there was no difference in reaction to BAL between rabbits and rats with severe renal damage developing two days after injection of uranium acetate and normal animals. Mortality rates were closely in agreement in both series so they concluded that the presence of serious renal disturbance does not alter the LD50 or lead to unexpected signs of BAL intoxication. Rabbits given BAL three and four days after injection of uranium acetate showed somewhat decreased tolerance for BAL. Thus 50% of the animals in both groups died after 60 mg. BAL/kg. as against a fatal level of 80 mg./kg. with normal animals. The LD50 has thus been decreased a little by the damage to the kidneys.

With a view to get [REDACTED] a more precise idea about the excretion of BAL or a BAL derivative in conditions of kidney damage, it was proposed to estimate the antimercury activity of urine obtained from/

from rabbits with kidneys damaged which were injected with 50 mg. BAL/kg. intramuscularly.

After preliminary test for blood and urine urea and urine albumin, 5 mg./kg. aqueous solution of uranyl acetate was injected subcutaneously into one male rabbit to produce renal damage according to Cameron, Burgess and Trenwith (loc. cit). After uranyl injection, blood and urine urea and urine albumin were tested to find the extent of renal damage. The observation is given in Table 18. Third and sixth day after the uranyl injection 50 mg.BAL/kg. with 0.5 ml. propylene glycol was injected intramuscularly into the rabbit and water 25 ml/kg. was given by stomach tube. Urine before and 2, 4 and 8 hours after the injection of BAL were collected by catheter and their antimercury activity was estimated as in the previous case. The results are given in Table 18.

TABLE 18/

Showing the amount of renal damage and the extent of antimercury in the urine of rabbit after intramuscular injection of 50 mg.BAL/kg.

Days	Weight of rabbit in Kg.	Urine in 24 hours	Blood urea mg. %	Urea gm. %	urine Albumin	Antimercury of urine.				
						2 hours sample	4 hours sample	8 hours sample	vol-ume	% of reversal
0	1.8	78ml.	28.5	3.47	nil	-	-	-	-	-
Uranyl acetate 5mg./kg. subcutaneously:										
2	-	76	-	-	nil	-	-	-	-	-
3	25 ml. water/kg. by stomach tube and 50 mg. BAL/kg. intramuscularly:									
	1.75	29	1.25	2.12	+	34ml	nil	nil	2.5ml	1.7ml nil
4	1.7	11	207	-	+++	-	-	-	-	-
6	25 ml. water/kg. by stomach tube and 50 mg. BAL/kg. intramuscularly:									
	1.6	3.2	16.7	nil	+++	31.2ml	nil	nil	2.6ml	-
7	Rabbit died in the evening.									

None of the samples of urine collected after the injection of BAL when added at a final dilution of 1:5000 to urease dilution showed any [REDACTED] anti-mercury effect either on the third or on the sixth day though there was progressively severe renal damage as shown by high blood urea and low urine urea and heavy precipitate of albumin in the urine. The rabbit died on the seventh day and on post-mortem the evidences of renal damage were detected.

The behaviour of uranyl acetate towards urease enzyme and to the reversal activity of BAL were tested in vitro and it was found that uranyl acetate when present up to 446.25 μ g. uranium ion in each unit, produced about 75% inactivation of urease activity and this inactivation could not be reversed even when BAL was added up to 2 mg. in each unit.

Estimations for antimercury activity present in the rabbit's plasma were made after intramuscular injection of 50 mg. BAL/kg. in experimentally kidneys damaged rabbit as on previous occasion. It was observed that 0.1 ml. undiluted plasma obtained from uranyl treated rabbit behaved more or less like a normal rabbit, i.e., it potentiates the urease activity/

activity up to about 13%. Diluted plasma (1:1250) either when obtained before or 30, 60 and 120 minutes after the intramuscular injection of BAL, neither produced any inhibition of urease, nor did any one of those show any reversal phenomena when added to 1:5 mercury poisoned urease. Nitroprusside colour reaction with a mixture of uranyl ion 3 mg./ml. and BAL 5 mg./ml. gave a negative result. So it is conjectured that BAL is being converted into some stable compound by uranium leaving no free -SH group active to give these tests positive.

VIII. Discussion

Conway and O'Malley's microdiffusion method (1942) has been adopted. Urease enzyme as glycerine extract of soya bean flour has been used mostly throughout the investigations, besides urease tablets B.D.H., which were also used for a few months. All these enzyme preparations were tested for their potency of ureolytic activity on a known amount of urea. 1:5 dilution of urease glycerine extract of soya-bean when allowed to act on 240 μ g. of urea for 30 minutes was found to give a constant hydrolysis which ranged between 75 to 168 μ g. urea (32 to 70% of the total urea used) according to the temperature and preparation used (Table 3). This dilution of urease was found most convenient. As will be clear from Tables 1 and 2, with undiluted urease the ammonia evolved in 15 minutes was equivalent to the urea present up to 180 μ g. and above this level the hydrolysis was incomplete. Of the various dilutions of urease when allowed to act on 240 μ g. of urea for varying lengths of time (Table 2), 1:5 dilution of urease when allowed to act for 30 minutes, yielded the maximum ureolytic activity.

The/

The temperature relationship with ureolytic activity of urease, as pointed out by Van Slyke and Cullen (1914) and Conway (1939) necessitated to maintain a record of temperature at which the experiments were performed.

As Van Slyke and Zacharias (1914); Kay and Reid (1934) and Conway (1939) reported that the optimum pH for total recovery of ammonia by the interaction of urease and urea is somewhat to the alkaline side of the neutrality, so the pH of urease dilution alone or when combined with other ingredients were maintained between 7 to 7.5 by adding 0.1 ml. of phosphate buffer pH 7.2 to each 1:5 dilution of urease.

It has been pointed out in the main text of the work that the inactivation of urease by heavy metals is an old established fact. Throughout this work, mercury has been used for poisoning the urease activity to the extent of 70 to 81%. In the beginning Hg. and arsenite were tried for inactivating the urease, but it was found that arsenite is too poor a poison, probably mercury having special affinity for urease. Similar type of observation was reported by Barron, Miller, Bartlett, Meyer and Singer (1947). They found lewisite a much more better/

better poison than arsenite for succinoxidase - a sulphhydryl enzyme (Hopkins and Morgan, 1938). Mercury 0.25 to 0.5 μ g. was required for this purpose depending on the preparation and the source of soya bean from which the urease were prepared (Table 5). An inactivation of 70 to 81% of the total urease activity has been selected as reversal of inhibition might become increasingly difficult when the concentration of the inhibitor was raised so as to produce complete inhibition. With this range of inactivation the reversal activity by BAL was found constant, i.e., for 1mM Hg 0.5mM BAL was needed.

Arsenite, though, proved about 80 times less potent poison for urease, showed another interesting thing i.e., when the urease was inactivated by arsenite up to 75%, BAL could not reverse this inactivation even when pure BAL was added to it. This lack of reversal of arsenite poisoned urease was probably due to the fact that arsenite might fix up the -SH group of urease, which could not be replaced by the -SH group of BAL when made available for substitution. In other words, the dissociability of -SH group of urease after it has gone into/

into combination with arsenite is probably not possible.

For the reversal phenomena two dithiols (BAL and mannitol-6-BAL ether), two monothiols (thioglycollic acid and cysteine hydrochloride), triacetyl BAL and l-cystine were employed. Out of these, BAL proved to be most potent, triacetyl BAL the least, while l-cystine could not reverse at all. The relative potency as calculated on molar basis for 25% reversal is as follows:

0.1 mM BAL = 0.14 mM mannitol-6-BAL ether
= 0.16 mM cysteine hydrochloride
= 4 mM thioglycollic acid
= 44 mM triacetyl BAL.

Though Fischer (1943) pointed out that the inactivation of urease by heavy metals run parallel to the -SH group of the urease, recently there has been a good deal of discussion about the kind of linkage formed between the heavy metal and the -SH groups of the protein molecule. The most probable view is that two types of linkage between the heavy metal and -SH of the protein group are possible -

(1) the heavy metal (M) may combine with the two

-SH groups: Protein $\begin{array}{c} \text{S} \\ \diagup \quad \diagdown \\ \text{S} \end{array} \text{M}$ forming a cyclic compound; //

compound;

(ii) it may combine with only one -SH group: protein-S-MR¹, as seems to happen with organic mercurial complexes. If only the first reaction occurs, the dissociation constant of the reaction between the -SH groups of the protein and the metal would be obtained from the equation

$$K = \frac{(-SH)^2 (M)}{(M \begin{array}{c} \swarrow S- \\ \searrow S- \end{array})}$$

while the second type of compound would give rise to the dissociation constant

$$K = \frac{(-SH) (MR^1)}{(M - S - R^1)}$$

If both the reactions took place, the two equations would have to be extended and the contributions of the one or the other would be difficult to determine. There is, however, indirect evidence in favour of the fact that heavy metals might combine with -SH groups of the protein, forming metal cyclic compounds (Barron and Kalnitsky, 1947). This view is further supported evidently by the rapid reactivation of heavy metal inhibition on addition of dithiols (BAL and mannitol-6-BAL ether) while monothiois/

monothiols (thioglycollic acid and cysteine hydrochloride) are poor agents for reactivation. Triacetyl BAL showed poor reversal quite probably due to traces of BAL present in it.

When mercury was allowed to act on urease for 30 minutes before BAL was added, the reversal was nil by up to 0.06 μ g. of BAL. This is probably due to the fact that when -SH group of urease has entered into some stable combination with mercury, the recovery or replacement of this -SH group by the -SH group of BAL is not possible. Similar observation was recorded by Stocken (1945) who could not reverse Hg poisoning in experimental animals when BAL was given 20 minutes after the mercury administration.

While making an attempt to estimate the anti-mercury effect of plasma after the intramuscular injection of BAL, it was found that plasma of normal rabbits interfered much with the mercury poisoning of urease, i.e., 0.1 ml. of undiluted plasma when added to 5 ml. of 1:5 dilution of mercury poisoned urease, it prevented about 82 to 88% of the mercury poisoning while at the same time it potentiated the urease activity up to 9 to 14%. So an attempt was made/

made to see the effect of repeated small doses of Hg. on the plasma. It has been observed that the potentiation of plasma is abolished only after 6 mg. Hg/kg. in rabbit and this could not be further poisoned even on pushing the mercury up to 9 mg./kg. when the rabbit died. Further, it has been observed that the potentiation of the poisoned plasma in the rabbit did not return even in a month's time. At this stage, the rabbit was treated with BAL for two days and on the fourth day no change was observed in plasma. After about $2\frac{1}{2}$ months from the initial poisoning, the normal potentiation activity of plasma was again observed. All these suggest that repeated injection of small doses of Hg. produced in plasma some permanent change which could only recover after the regeneration or replacement of newly-formed ingredients of plasma. Ofcourse, this experiment is not conclusive, but it suggests that further work may throw more light on this aspect.

As the final dilution 1:1250 of plasma did not produce any marked effect on urease or mercury poisoned urease activity, so this dilution was employed for studying the fate of BAL in blood after intramuscular injection of 0.4 mM BAL/kg. in rabbits. One interesting phenomenon noted at this stage was that when diluted/

diluted samples of plasma obtained after the injection of BAL was allowed to act for two minutes on the mercury poisoned urease, none of the post BAL injection samples of plasma showed any reduction of mercury inhibition, but when allowed to act on the same for 15 to 60 minutes, all the post BAL injection samples of plasma collected between $\frac{1}{2}$ to 3 hours showed constant varying degrees of reduction in mercury poisoning. The optimum time required for this reaction was about 15 minutes while if kept together for even 60 minutes it did not affect the degree of reversal any more. The plasma BAL mixture either immediately after mixing together or after incubation for 30 minutes, when added to mercury poisoned urease behaved in a similar way and there was no difference in % of recovery of BAL from such mixture either when allowed to act for about 2 minutes or 15 to 30 minutes. So this behaviour of antimercury activity present in BAL injected rabbits' plasma is thought to be due to some change brought about in BAL during the process of absorption. This inference requires further confirmation. The relative power of absorption of various thiols after intramuscular injection in rabbits have been shown in Figure XI. BAL seems to attain/

attain the highest concentration and also remains longest in the plasma. Thioglycollic acid, cysteine and mannitol-6-BAL ether being injected in aqueous solution are probably rapidly absorbed and rapidly excreted.

As Cameron et al (1947) reported, the possibility of detoxification of BAL by liver, experiments were performed with 20 mg. BAL/kg. in normal as well as in experimentally liver damaged rabbits. It seems likely that liver plays some part in detoxification of BAL as shown by the presence of antimercury activity in higher amounts in liver damaged rabbits (17.3 to 24.3% reversal in 60 minutes samples of plasma) in normal rabbits (1.4 to 5.1% reversal in 30 minutes and 1.4 to 2% reversal in 60 minutes samples).

Detection of antimercury activity in rabbits' urine without or with intramuscular injection of BAL has been tried. Initially, the urine collected from metabolism cages was tried, but it interfered much with the urease as well as with the mercury poisoned urease activity probably due to the contamination with metals of the metabolism cages. So catheter specimen of normal rabbits' urine was tried, but it also interfered much with the mercury poisoning and also with the urease activity like normal plasma. Various dilutions/

dilutions of such normal urine from 1:10 to 1:80 have been tried, but no two samples of urine obtained even from the same rabbit on different occasions gave different results, probably depending upon the dilution of urine due to intake of water by the rabbit. 1:100 dilution, giving a final dilution of 1:5000 with the usual diluted urease of rabbits' urine kept on standard laboratory diet even without water in their cages, did not affect either the urease activity or the mercury poisoning. So this dilution 1:5000 has been employed for investigation during this part of the work. Recovery of BAL from the mixture of BAL and urine, of the order of 0.05 μ g. BAL in each unit, after incubating for $\frac{1}{2}$, 1, 2 and 4 hours have been tried. It has been found that recovery of BAL from $\frac{1}{2}$ hour sample was about 100%, 1 hour 83%, 2 hours 26%, while there was no recovery of BAL from 4 hours sample.

Estimations of antimercury activity in samples of rabbits' urine collected 2, 4 and 8 hours after the injection of BAL, were made with or without water diuresis and it has been observed that in both cases the excretion of antimercury substances in the samples of urine collected after the injection of BAL were/

were the same, i.e., about 6% of the total amount of BAL injected. So it appears likely that BAL is excreted through the renal tubules.

While attempting to estimate the antimercury activity in urine of rabbit whose kidneys were damaged by uranylacetate, this method did not succeed as probably uranylacetate present in the system of the experimentally poisoned rabbit was inactivating the BAL as shown by the negative nitroprusside colour reaction.

In the light of the work done and results obtained, it is evident that this method is not specific for dithiols, though it is much sensitive and delicate, but it seems likely that further work with other compounds of arsenic or with other metals, may prove to be more useful in working out a specific method for estimation of dithiols only in vitro and in vivo.

IX. Summary

(1) A new method for estimation of BAL in vitro and in vivo is described depending on the enzyme disinhibition method.

(2) The method is sensitive and delicate and can detect BAL up to a fraction of $\mu\text{g.}$ depending on the urease employed.

(3) The method is not specific for dithiols as monothiols thio-glycollic acid and cysteine hydrochloride also give positive test.

(4) The method is equally applicable for estimation of thiols in biological fluids, e.g., in blood and urine.

(5) Evidence has been obtained that normal plasma contains -SH groups which interfere with the poisoning activity of mercury.

(6) The fate of various di- and monothiols in blood has been studied after intramuscular administration of various thiols in rabbits.

(7) Further evidence in support of detoxifying capacity of liver for BAL has been put forward by comparing the blood concentration of BAL (20 mg./kg.) in normal and in liver damaged rabbits.

(8) Excretion of BAL in urine after intramuscular injection of 50 mg.BAL/kg. has been studied, and/

and the possibility of tubular excretion of BAL in experimental animals has been put forward.

(9) The excretion of BAL in renal damaged rabbits could not be studied by this method as uranyl ion employed for renal damage inactivates BAL.

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My thanks are due to Dr. M. Weatherall for his kind help and advice throughout the work and in the preparation of this paper.

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